INVESTIGATIONS OF POLYMORPHIC COLOUR VISION IN NEW WORLD PRIMATES

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Abstract

Primate colour vision in New World primates is intriguingly complex; show a polymorphism where males are obligatory dichromats (i.e. perception similar to colour-blind humans that cannot differentiate red from green) Females can be either dichromats or trichromats (vision similar to normal humans). The role of such polymorphism remains unclear; however, two often tested hypotheses are related to predator detection and the locating of specific food resources. Here we investigated behavioural changes in male and female primates relating to the colour vision phenotypes and niche divergence as an adaptive feature responsible for maintaining colour vision polymorphism. For polymorphic colour vision to be complementary advantageous, primates should be able to perceive the behavioural changes resulting from sensory abilities of conspecifics. Cooperation tests were used with captive primates to investigate the possibility of primates recognising the visual ability of other individuals in the group. A molecular study of medium-long wavelength sensitive opsin alleles in Pitheciidae indicated a greater variation than reported in the literature and such complexity might increase the number of females with trichromatic colour vision. From a geographical analysis of South American primates, we found that primates avoided areas with high predator richness; however, species that possessed more complex colour vision systems were unaffected by predator richness. Thus, colour vision might be related to complementary advantages in having different colour vision systems in the same group (again increasing the probability of trichromats). Alternative methodologies (i.e. Machine Learning and Computer Vision) were employed to investigate the fitness of different phenotypes in detecting camouflaged targets showing that, contrary to from traditional hypothesis of advantages of dichromatic colour vision, trichromatic colour vision models are best suited for breaking through camouflage. A proof of concept to improve the collection of behavioural data and to investigate the role of cooperation for the maintenance of polymorphic colour vision is also presented. In conclusion, colour vision polymorphism in New World primates enhances the visual abilities of primate groups and is maintained by niche sexual diversification.

Chapter 1 - General Introduction

Senses are the link between the organism and its environment; they are the product of millions of years of evolution and thus, diverse in many aspects. Perceived stimuli can be chemical, electrical, magnetic, mechanical, acoustic, visual; light sensitivity is one of the most relevant senses in the animal Kingdom. Almost every living creature has a form of light detection (i.e., phototaxis or vision). Notably, an imaging forming eye appeared around 540 million years ago (e.g., trilobites) and this valuable ability allowed visual complexity in the sight of predators and prey, which, with other features, led to an evolutionary 'arms race' (Lamb, Collin, & Pugh, 2007). For primates, visual areas represent half of their brain's neocortex (Van Essen, Anderson, & Felleman, 1992). Besides its biological significance, vision is a relative sense relying on perception and it depends on three factors: the light source, the object, and the viewer.

A notorious aspect of vision is the perception of colour. Visible light is composed of a spectrum with a narrow range of wavelengths, approximately from 390 nm to 780 nm for humans (Stevens, 2013). This visible spectrum ranges from red to violet and human perception is used as reference. However, the perception of visible light is even more diverse and complex. Some animals are able to perceive ultraviolet range of the spectrum, such as bees and birds. Other species, such as vipers, can perceive infrared using electromagnetic radiation as a thermal detector (Bowmaker, 1998; Stevens, 2013). Other animals, such as insects and birds, use polarized light, which refers to the direction of the light hitting the eye, to navigate (Stevens, 2013). Due to the importance and relativeness of this sense, the understanding of the viewer's perception is of fundamental importance to better comprehend the viewer's behaviour, evolutionary adaptations, and ecological role.

While most research investigates the importance of colour vision to detect food items, the importance of predators and sociality remains mostly unexplored (Buchanan-Smith, 2005; Pessoa et al., 2014). This report presents an investigation into why New World primates present highly polymorphic colour vision. Pitheciidae species, such as *Callicebus*, are largely uninvestigated. Yet, their high allelic diversity of opsins is known in the primate colour vision literature (up to 5 alleles; Jacobs & Deegan 2005; Bunce et al. 2011). However, studies investigating this diversity were undertaken with inbreeded captive individuals and with possible hybrids in the group, while a study in the wild failed to find all five alleles in the same population (Bunce et al., 2011; Jacobs & Deegan, 2005). Recently, red-faced uakaris, which present a conspicuous red social display, have been reported with a high allelic variation of opsins (Corso et al., 2016). Polymorphic colour vision is greatly sex biased, given that only females can present trichromatic colour vision in New World primates. In spite of this strong sex bias, behavioural differences have not been widely reported. For instance, the advantages of dichromatism and trichromatism are well known (Table 1); however, the role of each phenotype within a group is not understood. Comparing the opsin diversity and consequently the proportion of trichromatic females in the group would help to elucidate the social role of trichromatic colour vision. To this end, we evaluated role of socio-sexual communication by evaluating a species with a strong red display (i.e., undetectable by dichromats; Sumner & Mollon 2003). It is expected that if the socio-sexual communication through reddish displays were responsible for driving the evolution of trichromatic colour vision, these species should present an enhanced colour perception and will not be polymorphic (i.e., not routinely suffer from any type of colour blindness as is the case with the majority of New World Primates; Jacobs 2007). As aforementioned, both *Callicebus* and *Cacajao*, share a highly polymorphic colour vision; however, data are lacking in other genera in this family (Bunce et al., 2011;

Corso et al., 2016; Jacobs & Deegan, 2005). Here we assess the allelic diversity in several Pitheciidae species (samples from the wild) and find support for high allelic diversity and its aforementioned consequences.

Part of the reason why colour vision and group cooperation is not well understood is that many variables are involved in the perception of colour in the wild. Illumination is highly variable in the forest canopy and throughout the day (Koop & Sterck, 1994; Théry, 2001). Moreover, the difficulties of working in the field, leads to small sample sizes and a paucity of data on group behaviour. While working under laboratory conditions would help to control the variables such as light levels, these would prevent the detection of relevant group interactions. Here we investigate the behaviour of males and females, in a field study, inferring the implications of different colour vision phenotypes. By measuring forest height use, food item colour, and time of the day, we investigate behavioural changes related to colour vision polymorphism and its relation to group behaviour.

Table 1: Summary of the potential benefits of trichromacy and dichromacy in mammals

CONCEPT	HYPOTHESIS
Benefits of trichromacy	
Frugivory[1]	Trichromats are superior in finding ripe fruits against a green background. Fruit consumption is responsible for the evolution of trichromacy
Folivory[2]	Trichromats are superior in detecting young red leaves against a green background. Leaves consumption is responsible for the evolution of trichromacy
Socio-sexual signals[3]	Discrimination of emotional states, socio-sexual signals and threat displays
Benefits of dichromacy	
Scotopic vision[4]	Better foraging and detection of predators in low light conditions
Camouflage detection[5]	Better performance in detecting (colour) camouflaged objects

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5- Morgan, M. J., Adam, A., & Mollon, J. D. (1992). Dichromats detect colour-camouflaged objects that are not detected by trichromats. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *248* (1323), 291-295.

Predation is a major selective force, which drives behavioural changes, population structure, and shapes ecosystems (Sih, Crowley, McPeek, Petranka, & Strohmeier, 1985). Such a strong force will have significant impacts on prey species' life history. Most research on primate predation is done by opportunistically registering predation attempts; yet, these attempts are rich enough to provide relevant knowledge on primate and predator interaction (Boinski & Chapman, 2005). In fact, predator risk is equally a selective force as predator itself, where the primate's perceived risk is especially relevant (Hill & Dunbar, 1998). One way of approaching the perceived risk and the predatory risk is to analyse the colour vision system present in geographic areas with different predator types and predator richness. To this end we mapped the distribution of predators and colour vision types (i.e., monochromatic, polymorphic, and routine trichromatic) throughout New World primates' species range, observing the effects

of the predation risk (i.e. predator species richness) on the local variation of colour vision phenotypes.

To further investigate the performance of colour vision phenotypes, it is important to understand if the signals emitted by predators, such as shape and colour, contain sufficient information to classify/identify a predator against a camouflaged background. Here we employ a computer vision algorithm to classify predator type and evaluate the algorithm's performance. We built a dataset of full trichromatic colour images and a second dataset with the same images, but modified to create colour blind image simulations (i.e., how they would be perceived by a red-green colour blind individual). Thus, the model will evaluate the performance of dichromatic and trichromatic image classifiers allowing conclusions about the abilities of different colour vision phenotypes in recognizing predators.

The implications of the polymorphic colour vision should be explored by assessing the cooperative foraging and vigilance where each colour vision phenotype is more suitable. To understand how primates interact in such scenarios will shed light into the evolutionary maintenance of this unusual visual system among primates. To do so, we present a proof of concept of a methodology to explore collections of live animals, such as in zoos, with the minimum interference on the keepers' routine. Therefore, increasing research in sensory ecology and enhancing behavioural research by facilitating the experimentation in zoos.

Experiments on colour discrimination have drawn scientific attention since 1800s. Lubbock (1889) summarised different experiments in his book and demonstrated that animals were sensitive to colours beyond the visible spectra, and reported that *Daphnia sp*. were affected not only by brightness, but by colours. Von Frisch in 1914 also demonstrated the colour vision of bees (Kelber, Vorobyev, & Osorio, 2003). For primates, in the early 1900s, a series of behavioural experiments with two rhesus monkeys and one male *Cebus* already indicated a "failure to react to the red" (Watson, 1909). However, the polymorphic colour vision of New World primates was first discovered in 1939 in the studies of Walter Grether, who found colour deficient vision in some South American primates and pointed to the sex-linked condition of colour blindness in humans (Jacobs, 1998; 2007). In the 30 years following Grether's pioneer research, few studies were done on colour vision of New World primates (De Valois & Jacobs, 1968; Jacobs, 1998). De Valois and Jacobs (1968) combined their expertise with Old World and New World monkeys, respectively, and concluded that these groups have considerable differences in their colour vision. The majority of research articles regarding mammalian colour vision were produced from the middle 1990s and the association between opsin genes expression and visual perception was the key reason for this recent increase (Bowmaker, 1998; Jacobs, 1998, 2010).

Presently colour vision research is composed mostly of physiology research focused on brain processing, neural pathways and photoreceptors. Behavioural studies with captive species require time and it has proven difficult to establish a standardised methodology for evaluating different species (Kelber et al., 2003; Martin & Bateson, 2007). Studies in natural environments are even more challenging (Samuni, Mundry, & Terkel, 2014; Zuberbühler, 2014). However, the number of studies in nature is increasing and they usually aim to determinate the principal selective pressures for colour vision evolution and understand

sensory ecology (Caine et al., 2010; Jacobs, 2010; A.D. Melin, Kline, Hickey, & Fedigan, 2013; Amanda D. Melin, Fedigan, Hiramatsu, & Kawamura, 2008)

Recent studies introduced a new approach in colour vision research by using gene therapy for colour blindness (Mancuso et al., 2009), and actively manipulating it through alterations of the opsin gene complement in knock-in mice (Jacobs, Williams, Cahill, & Nathans, 2007; Jacobs, 2010). Studies on colour vision have also been applied in clinical research for Parkinson's disease (Rodnitzky, 1998) and dyslexia (Vidyasagar, 1999). One of the major theories for dyslexia is the visual theory, which relates this disability to a visual impairment based on the division of two distinct neural pathways (Ramus et al., 2003).

To date, some aspects of colour vision are still not understood, for example, its evolution, function (Buchanan-Smith, 2005). Long-term studies and performance analysis on visual tasks in different ecological niches are contributing to our understanding of colour vision variation among primate individuals, populations and species (Buchanan-Smith, 2005).

1.1 Light, colour and vision

Light is an electromagnetic wave. It behaves as a wave when it propagates and as a particle when detected. This is known as duality. Einstein interpreted light as a stream of particles, called photons (Dimitrova & Weis, 2008). Cells in the mammalian eye called rods are sensitive to the point that they can detect a single photon (Rieke & Baylor, 1998). Visible light in humans is a narrow band of wavelengths from 800 nm to 400000000 nm in an electromagnetic spectrum that ranges from gamma-rays of 10 nm to radio waves of kilometres long (Land & Nilsson, 2012). Isaac Newton initially recognized light as colourless, and then colour perception only as a property of the sensory system (Newton, 1730). Thus, light is processed using two main properties. The first is the luminance, which is the achromatic signal. Achromatic information is important to distinguish brightness, limits, motion, depth, and small objects (Livingstone & Hubel, 1988). The second is colour, which is the capacity to contrast two parts of a light spectrum, independently of light intensity. We can imagine the achromatic signal as a 'line drawing' and the chromatic signal as a 'water painting'.

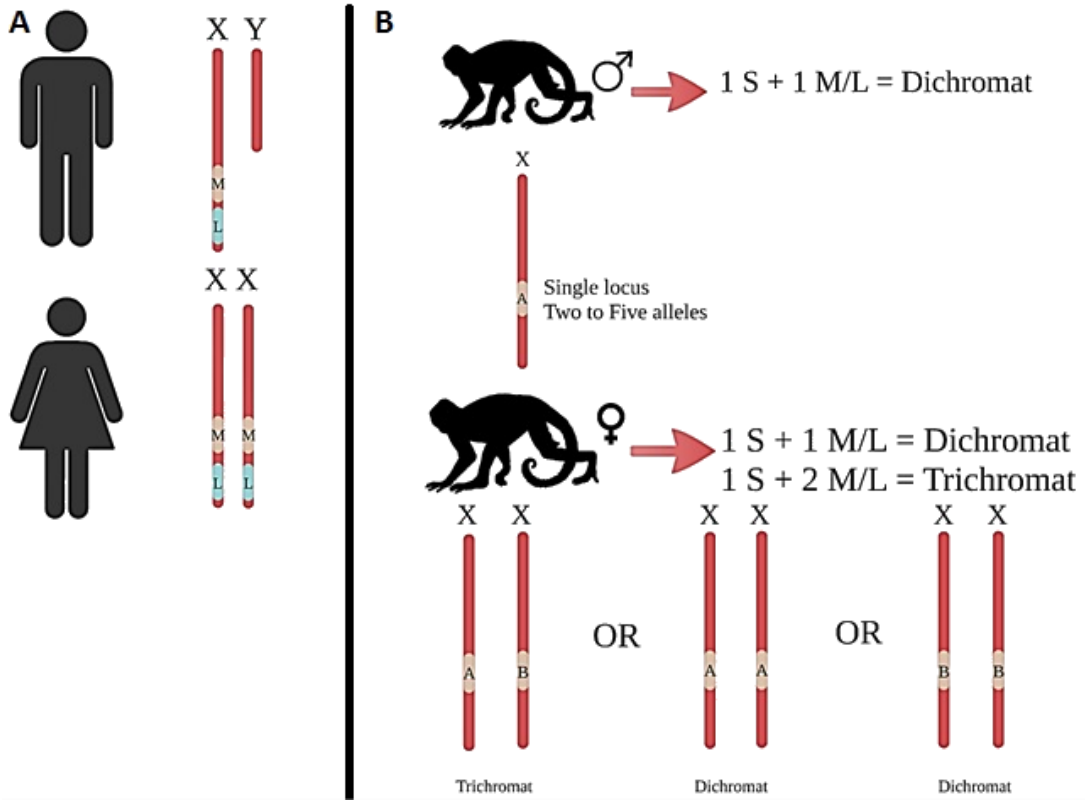
Light is detected by photoreceptors. Rods are evenly distributed on the retina and are related to scotopic vision (sensitive to low illumination), usually not participating in colour perception. Cones cells are denser at the fovea; they are less sensitive than rods and are classified according to their wavelength band, and mainly function in colour perception. Photoreceptors are composed of a carotenoid chromophore and a protein called opsin or rhodopsin (Land & Nilsson, 2012). Photopigments are sensitive to specific wavelengths of light (Jacobs, Neitz, & Neitz, 1993; Kelber et al., 2003). In summary, a chromophore captures photons, triggering the isomerization of the opsin. This activates a G-protein, causing an

enzymatic signalling cascade and an electrical response in the photoreceptor (Stevens, 2013). Colour discrimination requires at least two types of photopigments and neural mechanisms able to compare signals of photoreceptors in the retina (Rowe, 2002) and the presence of photoreceptors with different spectral sensitivities of sharp colour discrimination (Bowmaker, 1998; Kelber et al., 2003). The number of receptor types determines the array of colours, which the animal can potentially perceive (Stevens, 2013). The majority of mammals are dichromats, they possess two receptors with different peaks of sensitivity, which produce colour vision similar to red-green colour blindness in humans (Jacobs, 1990; Jacobs, Neitz, & Crognale, 1987; Mollon, Bowmaker, & Jacobs, 1984). Trichromats, as most humans and some primates, have three receptor types (Jacobs, 2009). Some fishes, amphibians, reptiles, and birds, have four classes of receptors (tetrachromats) for colour vision (Bowmaker, 1998). Coloured filters in the retina, such as oil droplets, can adjust the colour perception altering photoreceptor sensitivity. Some diurnal birds, lizards, fish and turtles have oil droplets in several cones, which enhance colour discrimination reducing spectral overlap from different cone signals (Bowmaker, Heath, Wilkie, & Hunt, 1997; Stevens, 2013; Vorobyev, 2003; Vorobyev, Osorio, Bennett, Marshall, & Cuthill, 1998). The mantis shrimp (*Neogonodactylus oerstedii*) has 12-15 different photoreceptors: it possesses an outstanding degree of colour vision complexity (Marshall & Oberwinkler, 1999; Thoen, How, Chiou, & Marshall, 2014).

There are five major types of visual pigments, which were present in early vertebrate lineages, before the origin of the major vertebrate classes (Bowmaker, 1998; Bowmaker, 2008). In fact, fishe, reptile and bird species are known to have four cone pigments and a rhodopsin, potentially creating tetrachromatic colour vision (Bowmaker, 1998; Bowmaker, 2008; Jacobs, Neitz, et al., 1993). Mammals have relatively poor colour vision due to a nocturnal common ancestor (Bowmaker, 1998). Nocturnal animals usually lose photoreceptors classes and invest

in rods, which are better adapted to vision in low light conditions (Bowmaker, 2008; Jacobs, 2010). Thus, a typical mammal's retina is densely populated with rods and only two classes of cones, as result they are likely dichromats (Bowmaker, 1998). However, this scenario has become more complex with new studies showing greater variation than expected among mammals. Some Australian marsupials appear to retain the ancestoral feature of medium-wavelength-sensitive photoreceptors, maintaining trichromacy (Arrese, Beazley, & Neumeyer, 2006; Arrese, Hart, Thomas, Beazley, & Shand, 2002). Furthermore, some nocturnal and marine mammals have lost the short wavelength sensitive opsin and are monochromats (Peichl, 2005). However, as previously stated, the assumption that eutherian mammals are mostly dichromats is still valid (Bowmaker, 2008).

About 35 million years ago, the primate line 'reinvented' colour vision, and they did it independently twice. Old World primates have duplicated the opsin gene for long and medium (M-L) wavelength on the sex chromosome X, which slightly shifts the peak of sensitivity, allowing routine trichromacy (Bowmaker, 1998; Jacobs, 2010; Jacobs, Neitz, Deegan, & Neitz, 1996). New World Primates do not have the routine trichromacy. Platyrrhini have polymorphic M-L opsin alleles for the same locus on the sex chromosome X, leading to a polymorphic colour vision in a single population (Jacobs, 2007). Where all males are obligatory dichromats, homozygotic females are dichromats and heterozygotic females are trichromats (Fig. 1) (Jacobs, 1996; Jacobs, 2007; Jacobs, 2010) (Figure 1).



X= Sexual chromosome X; Y= Sexual chromosome Y; S= Short wavelength opsin found at the autosomal chromosome 7; M/L= Middle/Long wavelength opsin; ♂= Male; ♀= Female.

Figure 1: Genetic basis of colour vision in Old World (A) and New World (B) primates

Polymorphic colour vision is an exceptional feature in primates' vision system. Insects are the most abundant and diverse taxa (Grimaldi & Engel, 2005). They have been subjected by opsin gene loss and gene duplication throughout their evolutionary history; however, polymorphic colour vision is not present in this group and they show low levels of intraspecific allelic variation (Briscoe & Chittka, 2001; Feuda, Marlétaz, Bentley, & Holland, 2016). Pollinating insects show a close relationship with the flowers they pollinate. Opsin sensitivities are fine tuned to identify colours in flowers and optimise pollination, which indicates the adaptive role of photoreceptors (Chittka & Briscoe, 2001; Chittka & Menzel, 1992). In the same way, some insects are highly cooperative species could cooperate employing polymorphic vision. Despite polymorphic colour vision not being observed in insects, the is paucity of research on

the subject and further studies are necessary to rule out polymorphism in insects (Briscoe & Chittka, 2001)

The polymorphic colour vision of the primate genus *Callicebus* is even more peculiar. Commonly, Platyrrhini species have two to three alleles for the M-L photopigments, while *Callicebus* have potentially five (Bunce et al., 2011). As consequence, the number of possible combinations of alleles rises from 6 to 15, and the number of potential heterozygote females also increases. This allelic diversity represents significant differences in visual perception (Jacobs, 1983, 1984) Also, differences between dichromatic individuals have received little attention (Jacobs & Deegan, 2005). For instance, two common types of colour blindness, protanopes and deuteranopes, have some variation in colour perception (i.e., difference of higher wavelength sensitivity of 6 nm) (Cole, 2004). With an increased number of M/L opsin alleles the number of trichromatic individuals increases proportionally with the combination of alleles. Great curiosity surrounds what selective forces are maintaining these polymorphism (Jacobs & Deegan, 2005). Among New World monkeys, two genera are the exception to this rule. The genus *Aotus*, the only nocturnal Platyrrhini is a monochromat (Colquhoun, 2006; Jacobs, Deegan, Neitz, Crognale, & Neitz, 1993). In the genus *Alouatta*, gene duplication, similar to that which occurred in the Catarrhini, has allowed routine trichromacy (Araújo et al., 2008). Furthermore, several genera have not yet been studied. For example, there are no data about colour vision on 50% of the genera of Pitheciidae family and this gap in knowledge will be addressed in this research project.

The evolution of trichromacy in Old World primates and the polymorphism observed in New World monkeys leads to the question: What is the adaptive value of colour vision? Finding food resources, detecting predators and observing social-sexual cues are all believed to be the main factors related to the benefits of trichromacy in primates (Table 1). However, other

mammals are well adapted to these tasks without such visual complexity: so why are primates so different?

In the following chapters we employed a multidisciplinary approach to tackle the problem of understanding polymorphic colour vision in New World primates.

In Chapter 2 we investigate the behaviour of males and females of a highly polymorphic species, *Callicebus nigrifrons* (Bunce et al., 2011; Jacobs & Deegan, 2005). Taking into consideration behavioural differences of males and females under daily light variation, we explore niche divergence as expressed by foraging behaviour, tree height use, and territorial behaviour. This is especially relevant as the maintenance of polymorphic colour vision relies on complementary advantages of each phenotype (Melin et al., 2012). Behavioural studies in the wild determining the role of polymorphic colour vision are of special interest as naturalistic conditions are difficult to replicate in captive studies and the challenges in performing research in the wild has led to a reduced number of publications, which explain the importance of colour vision. Unfortunately, we were not able to perform molecular analysis to identify the colour vision phenotype for each individual in the groups we studied due to low quality DNA and unreliable sequencing results. However, the behavioural data collected is robust enough to support conclusions on the role of different vision phenotypes in the groups studied.

In Chapter 3 we investigate how polymorphic New World primates are by molecular investigations of opsin diversity in an understudied family (i.e. Pitheciidae) (Bonci, Neitz, Neitz, de Lima Silveira, & Ventura, 2013) . The genera *Cacajao*, *Callicebus*, *Pithecia*, and *Chiropotes* are extremely variable in group size, body size and coloration (Mittermeier, Rylands, & Wilson, 2013). For instance, *Cacajao* features species with a very conspicuous red face and with white pelage (*Cacajao calvus)*, but also a cryptic black-faced counterpart with a

with mostly black fur coloration (Hershkovitz, 1987). By understanding the variation in opsins in this group, it is possible to infer the role of polymorphic colour vision in social signalling. For instance, are species with different social displays more polymorphic? Consequently, the data on allelic variation of medium-long wavelength sensitive opsins will support subsequent studies in this thesis by providing a bigger picture on the extent of variation in colour vision.

Predation risk is a major selective force shaping primate adaptations (Hart, 2007; Sih et al., 1985). In Chapter 4, we assess the impact of predator risk, measured by local predator richness, with monochromat, polymorphic, and trichromat colour vision primate species. Comparing already established relationships, such as body size and group size, with the response of colour vision types we analyse the strength of the response of primate genera towards predatory risk (Isbell, 2005). By analysing biogeographical patterns and correcting for phylogenetic signal, it is possible to explore the New World primate colour vision system as an adaptation for predator detection. Although primate and predator distributions are indirect evidence of predation risk, they possess relevant information regarding established relationships on a continental and phylogenetic scale. Monochromatic colour vision species should not be affected by a high predation risk, as they have a different approach to avoid predation risk (i.e., night activity). This allows the use of this group as a control group for subsequent analyses (Colquhoun, 2006; Hill, 2006). Therefore, understating which phenotypes are most affected by a higher predation risk will provide evidence for each colour vision type in relation to predator detection.

In Chapter 5, we investigate the importance of predator signals, such as shape and colour, which allow predator identification by dichromats and trichromats. Using computer vision and machine learning, we removed human error from classifying predators. A similar

approach has already been used for classifying cryptic and conspicuous primate food items, which was able to exclude human bias in the process (Melin et al., 2014). We further explore the performance of trichromatic and dichromatic trained classifiers in identifying camouflaged predators; thereby, demonstrating which vision type is most effective in break-through camouflage.

In Chapter 6, we present a proof of concept study to facilitate behavioural studies of vision perception in primates. Zoos are an important resource for scientific research; however, the increasing focus on conservation lead to difficulties in allowing the performance of pure research. By using methods that allows cause minimum impact on the keeper's routine and that does not require enclosure modifications, we facilitate sensory ecology hypothesis testing in captive animals. For instance, testing the importance of behavioural cooperation in zoo-housed primates under different scenarios to investigate the role of polymorphic colour vision.

In summary, here we discuss the complexity of polymorphic colour vision, the potential role of cooperation for maintaining polymorphic colour vision, the impacts of predator risk for different colour vision types, and using a novel approach the abilities of dichromats and trichromats to identify predators. This will support and guide future developments in understanding not only the advantages of each vision type, but also the role of polymorphic colour vision within primate groups and species.

Chapter 2 –Sex differences in behaviour and habitat use of wild titi monkeys

Abstract

Primates have marked sex differences in morphology, physiology, and social behaviour; however, the consequences of such differences are not well stablished for several groups. When compared with Old World primates, New World primates are less sexual dimorphic. Even though, males and females have distinct roles in infant care, group formation, and leadership. By investigating less sexual dimorphic species it is possible to identify causes of behavioural differences in primates. In this study, we investigated the behavioural differences between the sexes in *Callicebus nigrifrons.* Data on height in trees, canopy use, food colour and behaviour from four primate groups were recorded in a preserved Atlantic rain forest area. Males and females showed similar behavioural time budgets and foraged equally on different food categories. We found that sexes differed in tree height use with males staying higher than females. However, females were higher than males during the first hours of the day. In addition, females spent significantly more time at feeding sites when compared to males. We conclude that males and females have a distinct use of the canopy and it is marked during times where forest illumination is favourable for trichromatic colour vision phenotypes. Each colour vision phenotype will have a different perception of feeding sites, staying longer if it is perceived as richer in food. Trichromatic vision is best suited to identify ripe fruit, thus food patches will be assessed as of higher quality by trichromatic females and this explains why they stay longer. Here we show behavioural evidence of niche divergence between the sexes, which explain the maintenance of colour vision polymorphism in the investigated species.

2.1 Introduction

Sexual dimorphism in primates can result in significant behavioural changes. For instance, troop protection, social and behavioural roles are correlated with physical sexual dimorphisms (Pickford, 1986). In New World primates, diet also plays an important role in sexual dimorphism where folivores species tend to be more sexually dimorphic (Ford & Davis, 1992). It is known that females have different dietary requirements and metabolic rates due to pregnancy and lactation leading to a dimorphism in body weight, having males larger than females (Pickford, 1986). However, *Callicebus* do not have significant sexual dimorphism in body weight nor a diet based on leaves, consuming mainly fruits (Caselli & Setz, 2011; Hershkovitz, 1990). In terms of energy requirements between sexes and parental care in *Callicebus,* males are responsible for infant care and transport, whereas females are only responsible for nursing (Mendoza & Mason, 1986). Furthermore, this species makes a good model to compare sex differences in terms of vision since the species does not display any physical sexual dimorphism (i.e. males and females appear identical, except for external genitalia). Energy requirements for parental care, pregnancy and lactation are, probably, equalised. The most striking difference between males and females in *Callicebus* is the polymorphic colour vision, which can lead to an altered habit in finding food or detecting predators (Caine et al., 2010; Pessoa et al., 2014; Regan et al., 2001). *Callicebus nigrifrons* is a species with cryptic coloration and has little exposed skin (Hershkovitz, 1990). Therefore, by reducing the importance of socio-sexual colourful displays it is possible to investigate other variables further, such as foraging and predator detection, to explore the maintenance of polymorphic colour vision in this species (Changizi et al., 2006; Osorio & Vorobyev, 1996; Pessoa et al., 2014; Regan et al., 2001).

Usually, mammals possess dichromatic colour vision with a reduced ability to distinguish red-green colour targets (Bowmaker, 2008; Jacobs, 1993). Dichromatism is a result of a nocturnal mammalian ancestor that lost the photopigment (i.e. opsin) gene, thereby reducing colour perception (Bowmaker, 1998). Old World primates do have duplicated the gene for the medium-long wavelength sensitive opsin on the X sexual chromosome leading to a trichromatic colour vision, whereas New World primates do not (Jacobs & Deegan, 1999). However, mutations at specific sites of the medium-long wavelength sensitive opsin has allowed heterozygotic females to have two different alleles on each chromosome allowing trichromatic colour vision, whilst males having only one chromosome X are obligatory dichromats (Jacobs, Neitz, et al., 1993; Shyue et al., 1998). Polymorphic colour vision is a characteristic of New World primates and affects how they perceive their environment (De Valois & Jacobs, 1968; Hunt et al., 1998; Jacobs, Neitz, et al., 1993).

There are different advantages in having trichromatic or dichromatic colour vision. For instance, the detection of reddish socio-sexual displays, ripe fruits and tender leaves against a green background are easily achieved by trichromatic colour vision individuals, whereas vision in low light levels and breaking through camouflage (i.e. crypsis) are better achieved by dichromatic individuals (Caine et al., 2010; Changizi, Zhang, & Shimojo, 2006; Melin, Fedigan, Hiramatsu, Sendall, & Kawamura, 2007; Regan et al., 2001; Riba-Hernández, Stoner, & Osorio, 2004; Verhulst & Maes, 1998).

It is well established that trichromatic individuals have enhanced food detection abilities (Dominy, Garber, Bicca-Marques, & de Azevedo-Lopes, 2003; Osorio, Smith, Vorobyev, & Buchanan-Smith, 2004; Riba-Hernández et al., 2004). However, behavioural studies investigating social foraging are lacking to determinate whether or not this confers an advantage in terms of maintaining polymorphic colour vision in New World primates (Riba-

Hernández et al., 2004). In the best of our knowledge, studies to date did not find niche divergence in foraging time, tree height use or discriminate feeding sites between sexes in polymorphic species (i.e. trichromatic females versus dichromatic males) (Dominy et al., 2003; Melin, Fedigan, Hiramatsu, & Kawamura, 2008).

Colour is highly dependent on environmental light, which is variable under the forest canopy (Endler, 1993). For example, canopy light is composed of a mosaic of light gaps and shades changing contrast as each canopy layer decrease light intensity beneath it (Koop & Sterck, 1994; Théry, 2001). This has significant implications for colour perception in forest environments. Forest shade is dominated by greenish or yellow-green light with a richer proportion of middle wavelengths compared to direct illumination composed of the full spectrum of light (e.g. "white") (Endler, 1993). A viewer may identify the same colour differently given changes in the illuminant, or even viewers with different cone types could perceive the same colour and illuminant as different (Endler, 1990).

Primates have canopy strata preferences based on weight, food resources, predatory threats and sympatric species; for example, heavier primates are limited in the use of thin branches and have difficulties crossing gaps between trees (Cant, 1992). The sympatric Neotropical species *Cebus apella* and *Saimiri sciureus* have different use of the canopy reflecting their distinct diets, insectivorous for *Saimiri* in lower strata and frugivorous by *Cebus* in medium canopy levels (Fleagle, Mittermeier, & Skopec, 1981). As birds of prey are the main predatory threat to primates, smaller species usually dwell in lower strata levels, whereas larger species can occupy higher strata as prey body weight is a limiting factor for bird predation (Cunha, Vieira, & Grelle, 2006; Ferrari, 2008; Isbell, 2005). Morphological and ecological factors reflect the general pattern of canopy use by primates; however, sensory ecology is equally important

in understanding specific species' variations and informative for describing behavioural strategies.

Black-fronted titi monkeys, *Callicebus nigrifrons*, are a medium sized primate species weighing approximately 2 kilogrammes that, typically, live in family groups of an adult pair and usually up to three offspring (Byrne et al., 2016; Ferrari, 2008; Veiga et al., 2013) (Figure 2). Titi monkeys are highly social species showing strong social bond towards each other, spending great part of their active hours grooming or in physical contact with their tails intertwined (Anzenberger, Mendoza, & Mason, 1986; Clyvia et al., 2014; Fernandez-Duque, Valeggia, & Mason, 2000). Territories are established through collaborative duetting among individuals at the border of their area (Kinzey, Rosenberger, Heisler, Prowse, & Trilling, 1977; Robinson, 1981). This species features a highly diverse colour vision polymorphism system where males are obligatory dichromats (i.e. having vision similar to red-green colour blind humans) and females can possess either dichromatic or trichromatic colour vision (i.e. similar to normal vision humans) (Bunce et al., 2011; Jacobs, 1996; Jacobs & Deegan, 2005). *Callicebus* has six allelic variations for the medium-long wavelength opsin, probably, evenly distributed across the population, it is therefore predicted that 83.33% of females will possess trichromatic colour vision (VG unpublished data).

We predict behavioural changes between the sexes, where females will make the most of their enhanced colour vision perception (assuming they are trichromats) and thereby show a niche divergence from their social group males. However, some level of variation is expected since dichromatic females are statistically possible within our study groups. Tree height use will be different as the illumination is variable across the canopy strata; this variation in daylight penetrating the forest will determinate a different use of strata. The consumption of green and non-green food items will vary between sexes due to different perceptual abilities (i.e. it is predicted that females will consume more non-green food items).

2.2 Methods

2.2.1 Study area

The Reserva Particular do Patrimônio Natural Santuário da Serra do Caraça, which is an 11000 hectares private natural heritage reserve located in Minas Gerais, south-east Brazil, consists of a transition zone from Brazilian savanna (i.e., Cerrado) and Atlantic rainforest. The climate is composed of two marked seasons with a rainy period from October to March and a dry cooler winter from April to September (Cäsar, 2012; dos Santos, Galvão, & Young, 2012). Sample points were selected in areas with habituated study animals from research ongoing since 2003 (Cäsar, Byrne, Young, & Zuberbühler, 2012; Cäsar, Zuberbühler, Young, & Byrne, 2013).

2.2.2 Data collection

The study was conducted from January 2007 to January 2008 and November 2009 to November 2010 with a total of 12 individuals of *Callicebus nigriforns* sampled from four different groups. Trails established by previous research were used to follow the animals from dusk to dawn. Each group was followed at least one day per month. We used focal sampling of individuals for 30 minutes with instantaneous records every 5 minutes, alternating individuals during the day according to a Latin square design. Data on height in metres (using a rangefinder; Nikon model Forestry 550), GPS coordinates (Garmin 76 CSX), canopy level (i.e. ground, low, intermediate, canopy, and top), and behaviour (i.e. foraging, moving, sleeping site, social interaction, vocalisation, and resting) were recorded (Figure 3). Faecal samples were collected from all individuals and stored in RNA later to assess the colour vision phenotypes. The study was designed in a double-blind routine where the data collection and molecular analysis and statistics were performed independently.

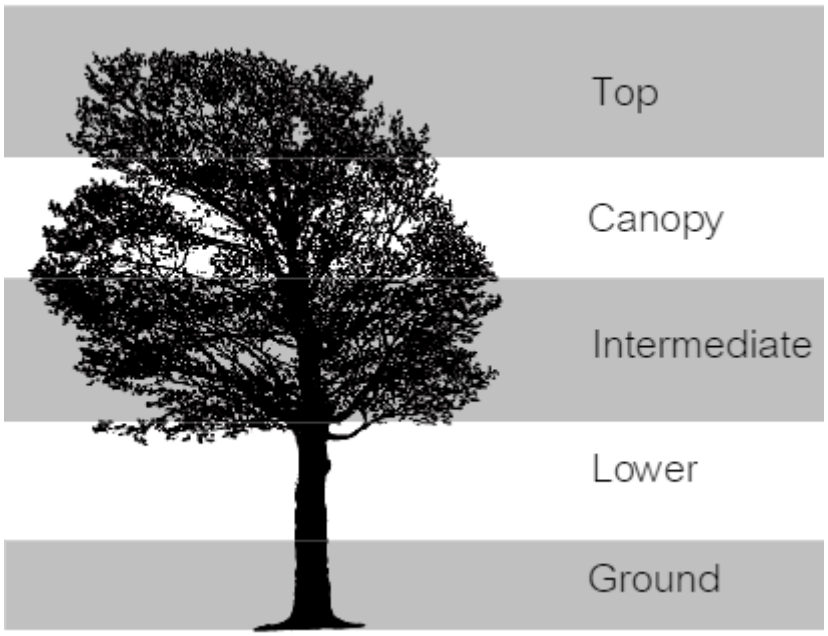


Figure 2: Categories of canopy levels recorded during behavioural observations

The study site is composed of a continuous patch of trees. For instance, the monkeys did not need to descend from a tree to move to another one. Thus, the preferred canopy level for expressing behaviours was chosen based on the natural behaviour of the species and not due to the configuration of the landscape. Food items consumed were recorded and colour classified as green or non-green (Yamashita, Stoner, Riba-Hernández, Dominy, & Lucas, 2005). Non-green food items were yellow, orange, brown, pink, purple and red. Along with fruit consumption, the time spent at feeding sites in seconds was also recorded. The feeding site was considered geographically independent from the food source; for example, at the same feeding site, animals would consume fruit, leaves and insects.

Behaviours were categorized as follows: foraging (when actively searching for food), manipulating food items or eating; moving (when the animal was in motion either vertically or horizontally in any tree strata); resting (when the animal is stationary paying attention to the surroundings or in a relaxed state); vocalizing (when the animal performed any type of vocalization, such as alarm calls or duets); social interaction (when the animal was engaged in interactions with other individuals in the group such as grooming); and (sleeping site when the animal reached the site where it will stay during the night).

2.2.3 Statistical analysis

First, a Chi-square test was used to analyse general differences in the behaviour of males and females, food type by sex, and food colour by sex. A Chi-square test was also used to compare the behaviour among females to check for discrepancies regarding the probabilities of polymorphic colour vision. This meant, checking if there were any females that behaved differently, which will be later related to the probability of dichromatic or trichromatic colour vision phenotype. We also performed a linear discriminant analysis to determine if males and females could be distinguished based on behaviours and height use.

A generalised linear mixed model (GLMM) was used to compare the canopy level and the tree height in metres to find if the trees varying in height have the same canopy level proportionally. For example, if the category top of the canopy is uniform in height across the study area. Groups were considered as random effect. A second GLMM was used to investigate the time spent at feeding sites and the fruit colour within the sexes having the amount of time at the feeding site as a response variable and sex nested for food colour as predictors in a GLMM: again with groups as random factors. An ordered logistic regression was used to model the effect of monthly precipitation, tree height use and hour by sex using groups as a random effect. To do so, we used the canopy level as the response variable and monthly precipitation, tree height in metres and hour of the day nested by sex as predictors. One female was removed from the behavioural analysis as it was not an adult. All statistical tests were performed using R (R Core Team, 2016) and MCMCglmm package (Hadfield, 2010).

2.3 Results

During the study, monkeys spent of their time 39.30% resting, 36.16% foraging, 17.99% moving, 3.65% in social interactions, 1.57% vocalising, 1.17% in the sleeping site (end of daily behavioural sampling) and 0.13% in other behaviours (Table 2). We found no difference in the behaviour expressed by male and females (χ^2=3.1781, DF= 6, P=0.7862). The most consumed food item was fruit (58.49%), followed by leaves (16.47%), flowers (13.75%) and insects (8.74%) (Table 3). We were not able to identify food items in 2.55% of feeding observations. No difference was found in the proportion of food type consumed by sex (χ^2=0.422, DF= 3, P=0.9356). Among females, one individual presented a different behavioural pattern with an increased proportion of resting and a decreased proportion of foraging behaving similarly to the males (χ^2=49.6927, DF= 25, P=0.0023). By performing a discriminant analysis, this one female was assigned more closely related to male behaviour and height use than to females (Predicted group = Male; True group= Female; Squared distance Female= 4.572, Male=3.130; Probability Female=0.327, Male 0.673). Even removing the female identified in the discriminant analysis, no difference is found in the behaviour of males and females (χ^2=3.746; GL=6; p=0.7110).

Table 2: Percentage of behaviours expressed from November 2009 to November 2010 by *Callicebus nigriforns* at the private natural heritage reserve Santuario do Caraca, Brazil.

Behaviour	Female	Male	All
Resting	34.37	43.49	39.30
Foraging	42.22	31.04	36.16
Moving	18.02	17.97	17.99
Social interaction	2.83	4.34	3.65
Vocalizing	1.31	1.80	1.57
Sleeping site	1.09	1.24	1.17
Others	0.15	0.12	0.13

Table 3: Percentage of food items consumed by *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil

	Female	Male	All
Flowers	13.12	14.26	13.75
Fruits	58.75	58.28	58.49
Insects	10.08	7.67	8.74
Leaves	15.78	17.02	16.47
NA	2.28	2.76	2.55

The tree height used by primates ranged from 0 (i.e. ground) to 26 metres from the ground with a mean tree height of 10.48 ± 4.63(mean ± SD). Tree strata use was mostly: canopy 55.78%, followed by the top of the tree 20.47%, the intermediate strata 18.02%, lower strata 5.45%, and ground 0.28%. By removing the ground strata due to very few observations and analysing the behaviours expressed in the different strata, we found that the top is used mostly for foraging, while resting, social interactions, vocalisations and sleeping sites most frequently under the canopy (Table 4). Females and males had a similar canopy level usage, however, males were overall higher in the trees than the females (Figure 4).

Table 4: Percent usage of tree strata from November 2009 to November 2010 by *Callicebus nigriforns* at the private natural heritage reserve Santuario do Caraca, Brazil.

Behaviour	Low	Intermediate	Canopy	Top
Foraging	6.47	8.60	45.93	39.00
Moving	3.35	20.45	61.34	14.87
Others	0.00	50.00	0.00	50.00
Resting	5.79	25.62	59.32	9.28
Sleeping Site	0.00	0.00	97.14	2.86
Social interaction	0.92	12.84	82.57	3.67
Vocalizing	2.13	17.02	74.47	6.38
All	5.45	18.02	55.78	20.47

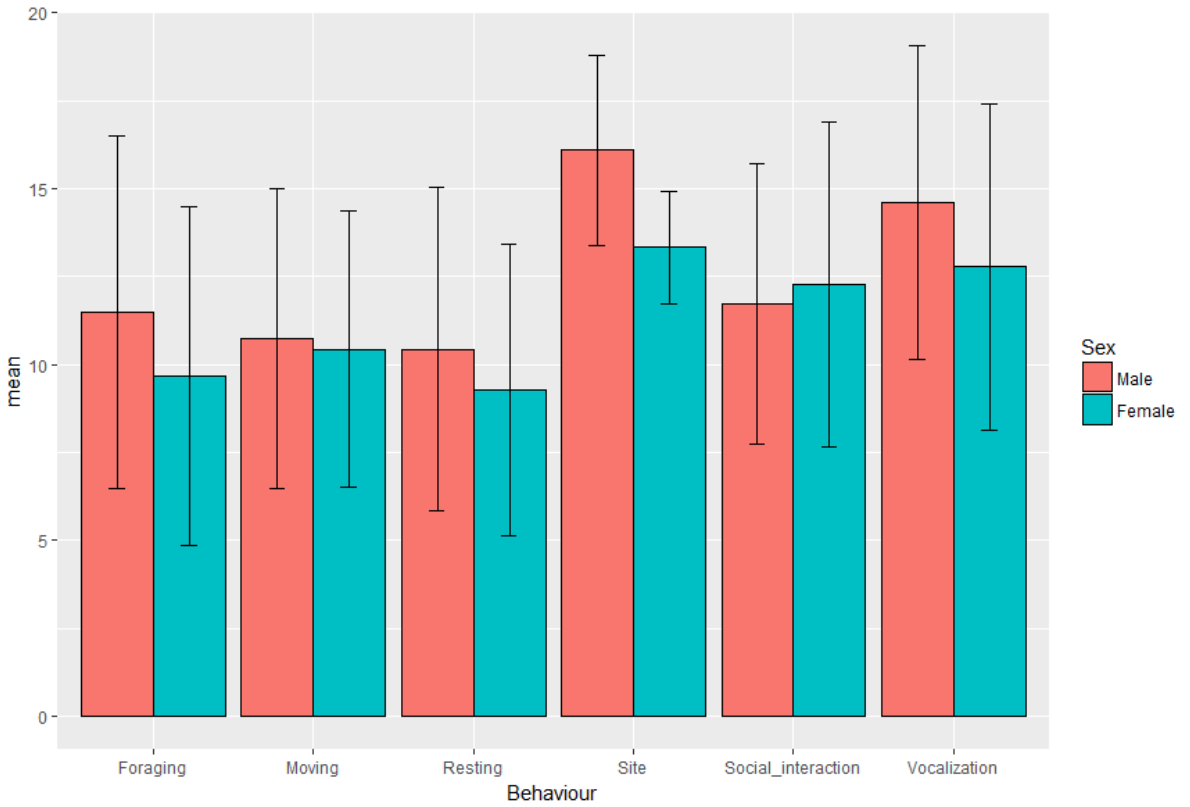


Figure 3: Behaviours expressed by mean tree height (m) by *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

By using a GLMM we found that foraging was significantly negatively related to tree height use, as were moving and resting. Vocalisations had a significant positive correlation with tree height use by *Callicebus* (predicting more vocalisations in the higher strata). As expected, sleeping sites were positively related to tree height. Although not significant, social interactions were negatively related to tree height use (Table 5).

Table 5: Generalised linear mixed models results for the effect of behaviour on tree height used by *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

	Coefficient	Lower 95% CI	Upper 95% CI	P Value
Random factor				
Group	1.9600	0.1716	5.2510	*
Fixed effects				
Intercept	12.4385	11.0234	13.9738	<0.001
Foraging	-1.9481	-2.7835	-0.9619	<0.001
Moving	-1.9208	-2.8137	-0.9967	<0.001
Resting	-2.5198	-3.4726	-1.6290	<0.001
Sleeping site	2.4103	1.5176	3.4190	<0.001
Social interaction	-0.7374	-1.6523	0.2124	<0.001
Vocalizing	1.3555	0.3608	2.2669	<0.001

CI = Confidence Interval; * = No P values given

However, when analysing tree height use variation during the day we found that females start the day higher than males and then go lower during the day. During the last hours of activity, females and males stay close to the same height finishing the day at the sleeping site (Figure 5).

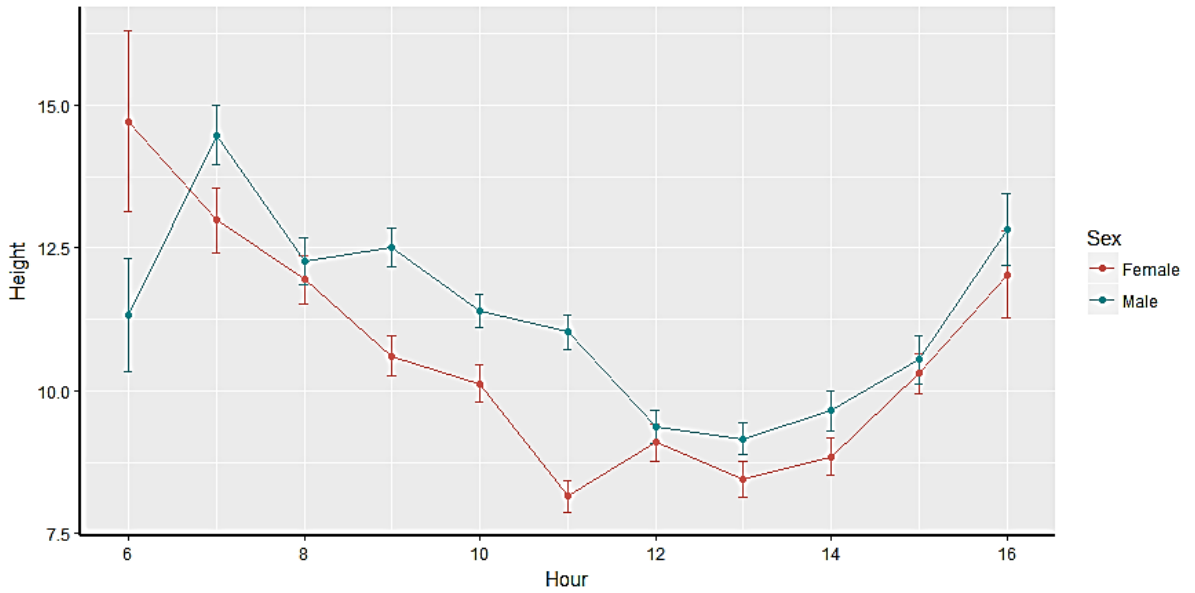


Figure 4: Tree height use variation per hour in *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

Since trees were different in height and considering the different study sites, we compared through a GLMM the tree heights in the canopy. We found that the canopy levels were significantly related to tree height across the study area and it is possible as to use this classification in subsequent analysis (Table 6).

Table 6: Results of the generalised linear mixed model for tree height (response) and strata (predictor) use by *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

	Coefficient	Lower 95% CI	Upper 95% CI	P Value
Random factor				
Group	0.0104	0.0008	0.0288	*
Fixed effects				
Low	0.9950	0.8604	1.1248	<0.001
Intermediate	1.9868	1.8983	2.0962	<0.001
Canopy	2.4023	2.3122	2.5026	<0.001
Top	2.5024	2.4233	2.6134	<0.001

CI=Confidence Interval; * = No P values given

Given that tree height was proportional to the canopy level across the study area, we investigated the odds ratio of finding *Callicebus* in the canopy level given the time of day, sex

and rainfall. We found no effect of rainfall on the use of the canopy. During the first hours of the day (i.e., 0700 hrs), the odds of having females moving in the canopy level is 25 times greater than at other time; this effect was not observed in males (Table 7).

Table 7: Ordinal logistic regression of canopy level by the time of the day, rainfall and sex for *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

	Coefficients	Std. Error	t value	Odds Ratio	P value
Intercepts					
Low|Intermediate	-3.0839	0.1693	-18.2128	*	0.0000
Intermediate|Canopy	-1.3884	0.1538	-9.0245	*	0.0000
Canopy|Top	1.2195	0.1532	7.9593	*	0.0000
Predictors					
Rainfall	-0.0009	0.0002	-4.0452	0.9990	0.9664
Females					
Hour 6	**-0.2078**	**0.3875**	**-0.5363**	**0.8123**	**0.0485**
Hour 7	**3.2525**	**1.0889**	**2.9869**	**25.8541**	**0.0006**
Hour 8	0.4084	0.2963	1.3784	1.5044	0.3402
Hour 9	0.6137	0.2247	2.7307	1.8473	0.4972
Hour 10	0.0220	0.2134	0.1032	1.0223	0.1792
Hour 11	-0.0457	0.2039	-0.2241	0.9553	0.2721
Hour 12	-0.4853	0.2084	-2.3287	0.6155	0.5086
Hour 13	0.0613	0.2204	0.2783	1.0632	0.9251
Hour 14	-0.3387	0.2125	-1.5940	0.7127	0.5878
Hour 15	**-0.1705**	**0.2136**	**-0.7981**	**0.8432**	**0.0001**
Hour 16	**-0.1916**	**0.2219**	**-0.8629**	**0.8256**	**0.0000**
Males					
Hour 6	**0.0199**	**0.2831**	**0.0702**	**1.0201**	**0.0000**
Hour 7	1.0149	0.7607	1.3341	2.7589	0.4729
Hour 8	0.8249	0.2725	3.0276	2.2818	0.0698
Hour 9	0.1517	0.2148	0.7063	1.1638	0.6934
Hour 10	0.2651	0.1944	1.3631	1.3035	0.4807
Hour 11	0.2894	0.1993	1.4519	1.3356	0.3529
Hour 12	-0.0807	0.1992	-0.4051	0.9225	0.0746
Hour 13	-0.3777	0.2068	-1.8264	0.6854	0.7669
Hour 14	-0.1238	0.2018	-0.6134	0.8835	0.8820
Hour 15	0.0464	0.3128	0.3128	1.0475	0.8820
Hour 16	-0.0675	0.2219	-0.3043	0.9347	0.8312

No difference was found in the amount of green and non-green food items consumed by sex (χ^2=1.290, DF= 1, P=0.2561) or food type consumed by sex; however, the time spent at the

feeding site was different for green and non-green food items between the sexes. Males spent less time feeding in green food feeding sites than females, and also females spent more time feeding in non-green food sites compared to males (Table 8).

Table 8: Generalised linear mixed model coefficients for the time spent at feeding site in seconds in relation to sex and food item colour for *Callicebus nigrifrons* in the private natural heritage reserve Santuario do Caraca, Brazil.

	Coefficient	Lower 95% CI	Upper 95% CI	P Value
Random factor				
Group	150.7	8.451	490.0	*
Fixed effects				
Female: Green	372.5	360.7	384.0	<0.001
Male: Green	361.7	349.9	1191	<0.001
Female: Non-Green	730.9	719.2	742.5	<0.001
Male: Non-Green	620.1	609.3	632.8	<0.001

2.4 Discussion

The tree height preference of *Callicebus nigrifrons* is for the "Canopy" category level level, which ranged from 10 to 14 metres from the ground, as found other studies (Trevelin, Port-Carvalho, Silveira, & Morell, 2007). The activity pattern of our study groups was similar to that reported for the species in other studies (Cäsar, 2012; Caselli & Setz, 2011). Fruits were the main food item consumed by *C. nigriforns*. Resting, foraging and moving were the main behavioural categories expressed and were similar in proportion when compared to reported data (Caselli & Setz, 2011; Trevelin et al., 2007). Males and females behaved similarly; however, they differed in tree height use by sex. Males were significantly higher in trees than females, except during the first hour of light. In the first hours of the morning the genera *Callicebus* leave their sleeping site to forage with males often the last to leave, thus corroborating our finding that females are higher during the first hours of the morning due to foraging behaviour (Kinzey & Becker, 1983).

Callicebus nigrifrons use a duet vocalisation to establish territories against neighbouring groups, these duets are a sequence of alternated vocalisations performed by males and females when in physical contact or very close proximity (Müller & Anzenberger, 2002). In the morning, a calling bout performed by males alone defines the location and proximity of neighbouring groups, if another group approaches, the female vocalises and duetting occurs (Robinson, 1981). Having males establishing the territory boundaries prevents other males from copulating with females or could provide an opportunity to copulate during intergroup encounters, which would contribute as a proximate cause for niche divergence in the species (Mason, 1966). Extra-pair copulations were recorded at the study site and are known to interfere in group social dynamics (Cäsar, 2012).

The lower canopy level is where most behavioural categories were expressed and this is related to predator avoidance, principally raptors, which are the main predatory threat to small primates (Bicca-Marques & Heymann, 2014; Ferrari, 2008). Sleeping sites are selected to provide the best protection against predators with animals spending the night at locations protected by entangled vines, closed canopy and exposed trunks that impede terrestrial predators from climbing (Bicca-Marques & Heymann, 2014). This explains the positive relationship of sleeping site height observed. Similarly, foraging, moving, social interactions and resting were more frequently expressed by monkeys when protected under the canopy.

Due to the highly polymorphic vision system found in *C. nigrifrons,* it is expected that a high number of females will have trichromatic colour vision in our study groups (Jacobs & Deegan, 2005, Goulart et al. Unpublish data). Having three to six opsin alleles in equal proportions would result in a 66% or 83.33% of trichromatic colour vision females in the groups, respectively. For instance, Bunce and collaborators (2011) found a proportion of 57.14% of trichromatic females in a study of wild groups of *Callicebus brunneus*, which possess three opsin alleles. It was not possible to assess the colour vision state in each animal as our molecular sequencing with the faecal samples collected failed (we did this analysis after behavioural data collection as we were using a blind experimental design); however, a general polymorphic state is expected for New World primates (Bunce et al., 2011; Jacobs, Neitz, et al., 1993). We report the fact that one female had behaviour similar to the males as a consequence of polymorphic colour vision. We suspect that this female had dichromatic colour vision phenotype and this explains her behavioural, foraging and tree height use similarity to males. As expected due to the proportions of opsin alleles in the species, one of the females would present a dichromatic vision. Based on the allelic frequencies found by Jacobs and Deegan (2005), 73.90% of females would be heterozygotes (i.e. trichromatic

colour vision). Therefore, we expected at least one of the studied females to have dichromatic colour vision (i.e., homozygote).

The importance of light levels is well established for the nocturnal genera *Aotus,* which has monochromatic vision (Colquhoun, 2006; Curtis & Rasmussen, 2006; Jacobs et al., 1993). Species will behave enhancing visual perception when expressing visual related activities such as foraging (Yamashita et al., 2005). The detection of ripe fruits is critically affected by the lighting where early or late in the day natural illumination will make orange-red fruits more detectable for trichromatic colour vision individuals (Endler, 1993). This would also explain why females were significantly higher in trees than males at the beginning of the day, then later males with their better low light (dichromatic) vision were higher (Endler, 1993; Yamashita et al., 2005). Unfortunately, we did not record vigilance behaviour to determine if this was related to tree height use. However, vigilant New World primates are usually lower when compared to non-vigilant individuals, as in lower levels threats from mammalian predators and ambushes from raptors are more probable (Smith, Kelez, & Buchanan-Smith, 2004).

Trichromats have a speed advantage in finding ripe food (i.e. red) quicker and more efficiently than dichromats (Smith, Buchanan-Smith, Surridge, Osorio, & Mundy, 2003). This is especially relevant under mesopic light levels found under forest canopy where trichromatic females are best suited for detecting fruit, insects and predators (Vogel, Neitz, & Dominy, 2007). Other studies have not found a foraging niche divergence by sex based on time used to find cryptic or coloured resources. Furthermore, trichromatic females are spatially further apart from the group as this represents an advantage for predator detection (Melin et al., 2008; Smith, Buchanan-Smith, Surridge, & Mundy, 2005; Smith et al., 2003). According to optimum foraging theory, animals will spend more time in patches with the highest perceived food

abundance (Pyke, Pulliam, & Charnov, 1977). Regarding time allocated at feeding sites, it is expected that animals will estimate the number of resources in a feeding patch, from their sampling behaviour, leaving the site when it has acquired the expected amount of food (i.e. hunting by expectation) (Krebs, Ryan, & Charnov, 1974). Therefore, dichromatic colour vision individuals (males) will perceive the environment differently from trichromatic colour vision individuals, spending less time at feeding sites because they perceive sites to be of lower quality than trichromatic females. This would explain our observations as to why females spend more time at feeding sites.

In summary, we provide behavioural evidence of niche divergence between males and females as a possible adaptive advantage in maintaining the polymorphic colour vision in New World primates. As discussed here, males were more attentive to encounters with neighbouring groups during the first hours of the day while females were using their enhanced colour perception to find foraging sites. Males made use of the higher light levels in the vertical stratification of the forest to improve their detection of objects by using light variation in the forest strata. The time allocated at the feeding sites by individuals appeared to be predicted by the perception of the patch quality, which depends on the type of vision possessed by individuals.

Chapter 3 - Medium/Long wavelength sensitive opsin diversity in Pitheciidae

Abstract

New world primates feature a complex colour vision system. Most species have polymorphic colour vision where males have a dichromatic colour perception and females can be either dichromatic or trichromatic. The adaptive value of high allelic diversity of opsins, a light sensitive protein, found in primates' eyes remains unknown. Studies revealing the allelic diversity are important as they shed light on our understanding of the adaptive value of differences in the colouration of species and their ecologies. Here we investigate the allelic types found in Pitheciidae, an understudied New World primate family, revealing the diversity of medium/long wavelength sensitive opsins both in cryptic and conspicuous species of this primate family. We found five alleles in *Cacajao*, six in Callicebinae (i.e. *Plecturocebus, Cheracebus,* and *Callicebus*), four in *Chiropotes,* and three in *Pithecia*, some of them reported for the first time. Both cryptic and conspicuous species in this group presented high allelic diversity.

3.1 Introduction

While most terrestrial mammals possess dichromatic colour perception, similar to a red-green colour blind human, primates may exhibit: 1) monochromatic colour vision in nocturnal species; 2) routinely trichromatic colour vision, mostly in Old World primates; and 3) polymorphic colour vision in most New World primates where males are obligatory dichromats, whereas females can have either dichromatic or trichromatic colour vision systems (Bowmaker, 1998; Jacobs, 2009). Strepsirrhine primate species have a potential polymorphic colour vision; however, this primate suborder lacks behavioural studies confirming a polymorphic colour vision as possessed by New World primates (Leonhardt, Tung, Camden, Leal, & Drea, 2009; Surridge, Osorio, & Mundy, 2003). This variation in colour vision in New World primates arises from a single locus for the gene coding the medium-long (M/L) wavelength sensitive opsin on the X chromosome (Jacobs, 2007; Jacobs & Rowe, 2004); that is, males are obligatory dichromats as they are hemizygotes, and females can be either dichromats (i.e., homozygotes) or trichromats (i.e., heterozygotes) (Jacobs, Neitz, et al., 1993). In New World primates, high allelic diversity of the medium-long wavelength sensitive opsin results in intraspecific variation in colour vision perception (Jacobs & Deegan, 2005).

The functional role of opsin polymorphism is still not completely understood; however, both trichromatic and dichromatic colour vision phenotypes are proven to have different behavioural advantages. Dichromatic individuals are better at detecting camouflaged stimuli and seeing in low light levels, whereas finding ripe food and new leaves are better detected by trichromats (Dominy & Lucas, 2001; Morgan, Adam, & Mollon, 1992; Osorio & Vorobyev, 1996; Regan et al., 2001; Verhulst & Maes, 1998). Social signals, observed on bare skin, such as emotional states, sexual signals, and threat displays are cited as factors leading to the

evolution of routine trichromatic colour vision (Changizi et al., 2006). Undoubtedly, bare skin is an important source of information, which might not be perceived by individuals with dichromatic colour vision. Even species that possess trichromatic colour vision might have difficulty to differentiate colours if the M/L opsin alleles in a heterozygotic female were separated by less than 10 nm (DeMarco, Pokorny, & Smith, 1992). Anomalous trichromacy (i.e., impaired colour vision but not complete loss) is frequent in howler monkeys and might be common in highly polymorphic colour vision species (Matsushita, Oota, Welker, Pavelka, & Kawamura, 2014).

Uakaries, genus *Cacajao*, are a Neotropical primate species occurring in the upper Amazonian region and they are represented by two contrasting colour phenotypes, one red-faced (i.e. *C. calvus*) and the other black-faced (*C. ayresi, C. hosomi, C. melanocephalus*) (Hershkovitz, 1987). Uakaris are a seed-eating specialists living in groups with up to 200 individuals in fission-fusion societies (Barnett & Brandon-Jones, 1997; Boubli et al., 2008; Bowler & Bodmer, 2009, 2011; Bowler, Knogge, Heymann, & Zinner, 2012). *Cacajao* was, until recently, a major lineage not investigated regarding its colour vision. It is now known that *Cacajao calvus* has high allelic diversity of opsins (Corso et al., 2016). The bare face of the bald red-uakari is thought to be related to communication among group members. Its simple one coloured face could allow for efficient communication through facial movements and expressions (Santana, Lynch Alfaro, & Alfaro, 2012). Importantly, the red colourful display on the bald uakari's face may present variations in haemoglobin pigmentation, which might indicate emotional states or health status, such as excitement and parasite infection (Changizi et al., 2006; Mayor et al., 2015). In addition, the bald uakari's vision was recently reported as being highly polymorphic with six functional alleles for the M/L wavelength sensitive opsin. This makes the species an interesting subject to test the importance of reddish displays on the evolution of colour vision.

Interestingly, the cryptic black-faced congeneric (i.e. *C. melanocephalus*, *C. ayresi* and *C. hosomi*) lack a bare face. To date, nothing is known about the colour vision on these species and this is one of the objectives of the present study.

The genus *Chiropotes*, also possesses species with red skin exposed on the face (*C. albinasus*), whereas other species in the genus do not possess this characteristic (Hershkovitz, 1985). These medium sized primates are secretive and difficult to observe in the wild; this is why there are few studies on their behaviour(Bicca-Marques & Heymann, 2013b). However, it is known that *Chiropotes* form fission-fusion groups with highly affiliative behaviour, and males are more gregarious and tolerant towards juveniles than females (Bicca-Marques & Heymann, 2013b; Veiga, Silva, & Ferrari, 2005). One *Chiropotes* species (*C. utahickae*) was investigated with regards to its colour vision and three M/L opsin alleles were found, also possessing a colour vision similar to other New World primates (Lima et al., 2015).

Pithecia is a species with cryptic colouration and lives in small group sizes (i.e., 4 to 6 individuals)*;* however, their diet is similar to *Cacajao* and *Chiropotes* (Barnett, Boyle, & Thompson, 2016). In the pitheciidae, the genus *Pithecia* presents the highest degree of sexual dichromatism (Bicca-Marques & Heymann, 2013b). This is a relevant factor when studying the polymorphic colour vision system in New World primates, especially considering the importance of this characteristic in relation to visual communication. This genus also possesses a polymorphic colour vision system as observed in other New World primates with three different known alleles for the M/L opsin gene (Lima et al., 2015).

In the Callicebinae, the genus *Plecturocebus* were also found to possess a relatively high number of alleles for M/L opsins. While most New World primates have three types of photopigments for the M/L wavelength, *P. moloch* have five cone types in a range from 530 to 562 nm (Jacobs & Deegan, 2005). Despite similarities in the higher allelic diversity, *Cacajao*

and *Plecturocebus* are contrasting in many ways. Callicebinae is the New World primate subfamily with the greatest number of species (33 recognised species) (Byrne et al., 2016; Mittermeier et al., 2013). Despite the number of species, it is one of the least studied primate subfamilies (Bicca-Marques & Heymann, 2013a). Titi monkeys are small (1.5kg) with timid behaviour spending most of the time under umbrella-like canopy, forming family groups commonly of five individuals with a diet consisting of leaves and fruits (Bicca-Marques & Heymann, 2013a; Mason, 1966; Veiga et al., 2013). Some species show countershading coloration with a bright coppery colour. It is assumed that this colouration is not visible to dichromatic colour vision individuals and the role of this conspicuous colouration is still unknown (Sumner & Mollon, 2003). Interestingly, only two species have been investigated so far regarding their colour vision: *Plecturocebus brunneus* and *Plecturocebus moloch.*

There are three main methods employed to determine colour vision perception: behavioural studies, direct physiological measurements and molecular analysis of opsin genes (Gomes, Pessoa, Tomaz, & Pessoa, 2002; Jacobs, Neitz, & Krogh, 1996). In the behavioural approach, animals are trained to select colour referenced stimuli in discrimination experiments, which evaluate the degree of difficulty to detect coloured stimuli (Gomes et al., 2002). In the physiological approach, an electroretinogram is performed where the spectral sensitivity of photoreceptors in the retina is measured or spectrophotometry of *in vitro* reconstituted photopigments from cDNA are measured (Jacobs, 2007). Molecular analysis is the most widely employed method. From molecular analysis, it is possible to infer the peak of sensitivity of the expressed opsin gene by verifying amino acid changes at specific sites (Shozo Yokoyama, Yang, & Starmer, 2008). The combination of both molecular and ecological data promises to provide new insights on the role of colour vision evolution in primates (Buchanan-Smith, 2005).

The maintenance of such a high number of alleles in this family, strongly suggests that polymorphisms has an adaptive function. Here we evaluate, qualitatively, the allelic diversity in the family Pitheciidae (*Cacajao*, *Plecturocebus, Cheracebus, Callicebus, Chiropotes,* and *Pithecia*).

3.2 Methods

3.2.1 DNA extraction

Genomic DNA was extracted from primate muscle tissue (Table 9) stored in 100% ethanol using DNeasy blood and tissue kit (QIAGEN) following the manufacture's protocol without modifications. The extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientific) to rule out allelic dropouts (i.e., sequencing one copy of the gene only in heterozygous individuals) by using samples with a DNA concentration higher than 200 picograms/microliter (Beja-Pereira, Oliveira, Alves, Schwartz, & Luikart, 2009; Morin, Chambers, Boesch, & Vigilant, 2001). We used 1 µL of extracted DNA in buffer AE to obtain 260 nm readings providing the DNA concentration, which were, on average 20000 pg/µl.

Table 9: List of species used in the molecular analysis of allelic types of Medium/Long visual photopigments

Species	Female	Male	Total
Cacajao ayresi	2	0	2
Cacajao calvus	2	4	6
Cacajao hosomi	1	2	3
Cacajao melanocephalus	2	1	3
Plecturocebus cinerascens	4	2	6
Plecturocebus bernhardi	2	2	4
Cheracebus lugens	8	3	11
Plecturocebus miltoni	1	2	3
Plecturocebus moloch	1	1	2
Callicebus nigrifrons	5	3	8
Chiropotes albinasus	1	0	1
Chiropotes israelita	3	1	4
Chiropotes satanas	0	1	1
Pithecia irrorata	2	0	2
Pithecia pithecia	0	1	1
Total	34	23	58

3.2.2 Sex Assignment

To confirm the primates' sex from all samples, Polymerase Chain Reactions (PCR) were conducted using the primers for the Amelogenin in the X chromosome (Forward: 5' – ACCACCAGCTTCCCAGTTTA – 3'; and Reverse: 5' – GCTGGGWTAGAACCAAGCTG – 3') for a ~200 bp fragment, and the Y-linked sex-determining region (SRY) (Forward: 5' – AGTGAAGCGACCCATGAACG – 3'; and Reverse: 5' – TGTGCCTCCTGGAAGAATGG – 3') for a ~165 bp fragment (Di Fiore, 2005). A 25 µL PCR was performed 0.25 µL of TaKaRa Ex Taq® Hot Start Version (1.25 units), with 2.5 µL of x10 Ex Taq buffer, 4 µL of 2.5 mM dNTP mixture, 0.8 µL of each primer at 100 pmol/µL, 3 µL of template DNA, and 14 µL of pure PCR water to complete the final volume. All reactions were performed with a negative control using pure PCR water instead of DNA template.

The thermal cycling profile followed one cycle of initial denaturation at 94°C for two minutes; then 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and

elongation at 72°C for 30 seconds; lastly, a final elongation cycle of 72°C for 5 minutes. Amplifications were confirmed by electrophoresis in a 1.3% agarose gel, using HyperLadder I™ (Bioline) as reference. The amplification of the Amelogenin, gene present in all samples at the X chromosome, was used as a positive control for all reactions and confirmed through molecular weight. The presence of two bands (i.e. one for the X and one for the Y chromosome) in the agarose gel allowed the assignment of males, while one band determinate females (i.e. one band for the X chromosome) through a benchtop verification (Di Fiore, 2005).

3.2.3 Amplification and sequencing

Primers for the Exon 3 were: Forward 5' – CTCCAACCAAAGATGGGCGG – 3'; Reverse 5' – ATCACAGGTCTCTGGTCTCTG – 3'. Primers for the Exon 5 were: Forward 5' – GAATCCACCCAGAAGGCAGAG – 3'; Reverse 5' – ACGGGGTTGTAGATAGTGGCA – 3'. PCRs were carried out using 0.25 µL of TaKaRa Ex Taq® Hot Start Version (1.25 units), with 2.5 µL of x10 Ex Taq buffer, 4 µL of 2.5 mM dNTP mixture, 0.25 µL of each primer at 100 pmol/µL, 0.5 µL of BSA (New England BioLabs) 3 µL of template DNA, and 14.25 µL of pure PCR water to a final 25 µL reaction. Negative controls were employed in all reactions using pure PCR water instead of DNA template. The Exon 3 thermocycling profile consisted of one initial cycle of 98°C for five minutes; 40 cycles of 98°C for 30 seconds, 62°C for 30 seconds decreasing 0.1°C per cycle, and 72°C for 30 seconds; followed by a final elongation cycle of 72°C for 5 minutes. The Exon 5 used the same thermocycling profile, but the annealing temperature was 60°C decreasing 0.1°C per cycle. PCR clean-up and sequencing were performed independently by Source

Bioscience Sequencing commercial service (Rochdale, UK) using Applied Biosystems 3730 series DNA Analysers.

3.2.4 Genotype determination

Amino acid changes at site 180 in Exon 3, 277 and 285 in Exon 5 are responsible for major shifts in the peak absorbance of the M/L photopigment and are known as the "three-site rule" (Hiramatsu et al., 2005; Yokoyama & Radlwimmer, 2001). For instance, a change at the site 180 from an Alanine for a Serine shift the absorbance peak in +7 nanometres; at the site 277, a Phenylalanine for a Tyrosine shift +8 nanometres, and at the site 285 a change of an Alanine for a Threonine shift the peak of absorbance in +15 nanometres. In the case of an opposite substitution, it is possible to subtract the constant for the site and obtain an approximate sensitivity peak. The site 294 is also known to shift the predicted peak in spectral sensitivity in Atelids and was also verified (Matsumoto et al., 2014). By examining these sites allows to identify six major types of photopigments found in New World monkeys. Sequencing from both forward and reverse strands from, at least, two independent PCRs were used to determinate the type of M/L photopigment. Fragments were edited and mapped to the reference opsin gene (GenBank NM000513) (Nathans, Thomas, & Hogness, 1986) analysed using Geneious (Kearse et al., 2012). Double peaks in the chromatogram were used to assign the individual as a homozygote, heterozygote, or hemizygote employing a specific plugin of Geneious software (Heterozygotes) (Kearse et al., 2012). Individuals assigned as double heterozygotes (i.e. double peaks at 180, 277, or 285) were not considered.

3.3 Results

All individuals had their sex confirmed by molecular analysis. By analysing the sites 180 at the exon 3 and sites 277, 285, and 294 at the exon 5 we were able to find six allelic variations of the M/L opsin gene (Table 10 and 11). We found five alleles in the *Cacajao* genus (532, 545, 550, 555, 560 λ_{max}nm). We found *C. ayresi* with the alleles AFA (532 λ_{max}nm), AYT (555 λ_{max}nm) and SYT (560 λ_{max}nm); *C. hosomi* with the alleles AFA, AFT (545 λ_{max}nm) and SYT; *C. melanocephalus* with AFT, AYT and SYT. These three *Cacajao* species have highly pigmented skin. *C. calvus*, which has exposed red facial skin, had four of these alleles, namely, AFA, AFT, SFT and SYT.

We found 6 alleles in Callicebinae (532, 534, 545, 550, 555, 560 λ_{max}nm)*: P. bernhardi* with AFT, AYT, and SYT; *C. cinerascens* with SYT, AYT, and SFT (550 λ_{max}nm); *C. lugens* with AFA, SFA, AFT, SFT, AYT and SYT; *P. miltoni* with SFT and AFT; *P. moloch* with AFT and SFT; and *C. nigrifrons* with SFT, AYT, AFT, and SYT. It was not possible to identify the allele in one individual of *C. lugens* due to double heterozygous sites at 277 and 285.

We found four alleles in *Chiropotes* (532, 545, 555, 560 λ_{max}nm): *C. albinasus* with allele SYT; *C. israelita* with the alleles AFA, AFT, AYT and SYT; and *C. satanas* with allele AFA. In *Pithecia,* three alleles (532, 545, 555 λ_{max}nm): *P. irrorata* with AFA, AFT and AYT and *P. pithecia* with AFT.

All sequences were identified from repeated sequencings from both strands and independent PCR reactions. Sequences are available in GenBank (Accession numbers KY345056-KY345113).

Table 10: Observed numbers of alleles of Medium/Long wavelength sensitive opsin in Pitheciidae primate species

Species	Allele	SYT	AYT	SFT	AFT	SFA	AFA
Expected λ_{max} nm		560	555	550	545	534	532
Cacajao	*ayresi*	1	1				1
	calvus	3		1	1		2
	hosomi	1			2		1
	melanocephalus	1	3		1		
Plecturocebus	*cinerascens*	4	3	1			
	bernhardi	1	2		1		
	miltoni			1	3		
	moloch			1	3		
Cheracebus	*lugens*	1	3	1	2	1	4
Callicebus	*nigrifrons*	4	6	1	1		
Chiropotes	*albinasus*	1					
	israelita		1		2		2
	satanas						1
Pithecia	*irrorata*		1		1		1
Pithecia	*pithecia*				1		

Table 11: List of species and medium/long wavelength sensitive opsins genotypes found in Pitheciidae

Species	Sex	180	277	285	294	Allele		Provenance
Cacajao ayresi	Female	A	F	A	N	AFA		Araçá River, Amazonas, Brazil
Cacajao ayresi	Female	A/S	Y	T	N	AYT	SYT	Araçá River, Amazonas, Brazil
Cacajao calvus	Male	S	Y	T	N	SYT		Sacado do Tarauacá, Acre, Brazil
Cacajao calvus	Male	A	F	A	N	AFA		Jutai River, Amazonas, Brazil
Cacajao calvus	Male	A	F	A	N	AFA		Jutai River, Amazonas, Brazil
Cacajao calvus	Female	A/S	F	T	N	AFT	SFT	Jutai River, Amazonas, Brazil
Cacajao calvus	Female	S	Y	T	N	SYT		Jutai River, Amazonas, Brazil
Cacajao calvus	Male	S	Y	T	N	SYT		Jutai River, Amazonas, Brazil
Cacajao hosomi	Male	S	Y	T	N	SYT		Serra do Imeri, Xamata, Amazonas, Brazil
Cacajao hosomi	Female	A	F	T/A	N	AFT	AFA	Serra do Imeri, Xamata, Amazonas, Brazil
Cacajao hosomi	Male	A	F	T	N	AFT		Salto Hua, Amazonas, Brazil
Cacajao melanocephalus	Male	A	Y	T	N	AYT		Amanã Lake, Amazonas, Brazil
Cacajao melanocephalus	Female	A	Y/F	T	N	AYT	AFT	Manacapuru, Amazonas, Brazil
Cacajao melanocephalus	Female	A/S	Y	T	N	AYT	SYT	Santa Isabel do Rio Negro, Amazonas, Brazil
Plecturocebus cinerascens	Female	S	Y	T	N	AYT		Aripuana, Amazonas, Brazil
Plecturocebus cinerascens	Male	S	F	T	N	SFT		Pimenta Bueno, Rondônia, Brazil
Plecturocebus cinerascens	Female	A/S	Y	T	N	AYT	SYT	Pimenta Bueno, Rondônia, Brazil
Plecturocebus cinerascens	Male	S	Y	T	N	SYT		Pimenta Bueno, Rondônia, Brazil
Plecturocebus cinerascens	Female	S	Y	T	N	SYT		Pimenta Bueno, Rondônia, Brazil
Plecturocebus cinerascens	Female	A/S	Y	T	N	AYT	SYT	Cabixi, Rondônia, Brazil
Plecturocebus bernhardi	Male	A	Y	T	N	AYT		Aripuana, Amazonas, Brazil

Plecturocebus bernhardi	Female	A	F	T	N	AFT		Aripuana, Amazonas, Brazil
Plecturocebus bernhardi	Male	A	Y	T	N	AYT		Aripuana, Amazonas, Brazil
Plecturocebus bernhardi	Female	S	Y	T	N	SYT		Machadinho D'Oeste, Rondônia, Brazil
Cheracebus lugens	Female	A	Y	T	N	AYT		São Gabriel da Cachoeira, Amazonas, Brazil
Cheracebus lugens	Female	A	F	T/A	N	AFT	AFA	Ig Mandiquie, Amazonas, Brazil
Cheracebus lugens	Female	S	F	T/A	N	SFT	SFA	Rio Marauia, Amazonas, Brazil
Cheracebus lugens	Female	A	Y	T	N	AYT		Balawau, Amazonas, Brazil
Cheracebus lugens	Female	A	F	A	N	AFA		Marari, Amazonas, Brazil
Cheracebus lugens	Female	A	F	A	N	AFA		Ig Anta, Amazonas, Brazil
Cheracebus lugens	Male	A	Y	T	N	AYT		Ig Anta, Amazonas, Brazil
Cheracebus lugens	Male	A	F	A	N	AFA		Ig Cuieiras, Amazonas, Brazil
Cheracebus lugens	Female	S	Y/F	T/A	N	*		São Gabriel da Cachoeira, Amazonas, Brazil
Cheracebus lugens	Female	S	Y	T	N	SYT		São Gabriel da Cachoeira, Amazonas, Brazil
Cheracebus lugens	Male	A	F	T	N	AFT		Marari, Amazonas, Brazil
Plecturocebus miltoni	Male	A	F	T	N	AFT		Aripuana, Amazonas, Brazil
Plecturocebus miltoni	Female	A/S	F	T	N	AFT	SFT	Aripuana, Amazonas, Brazil
Plecturocebus miltoni	Male	A	F	T	N	AFT		Aripuana, Amazonas, Brazil
Plecturocebus moloch	Female	A/S	F	T	N	AFT	SFT	Alta Floresta, Mato Grosso, Brazil
Plecturocebus moloch	Male	A	F	T	N	AFT		Alta Floresta, Mato Grosso, Brazil
Plecturocebus moloch	Male	A	F	T	N	AFT		Alta Floresta, Mato Grosso, Brazil
Callicebus nigrifrons	Male	A	Y	T	N	AYT		Minas Gerais, Brazil
Callicebus nigrifrons	Female	A/S	Y	T	N	AYT	SYT	Minas Gerais, Brazil
Callicebus nigrifrons	Male	A	F	T	N	AFT		Minas Gerais, Brazil
Callicebus nigrifrons	Female	A	Y	T	N	AYT		Minas Gerais, Brazil
Callicebus nigrifrons	Male	S	F	T	N	SFT		Minas Gerais, Brazil
Callicebus nigrifrons	Female	A/S	Y	T	N	AYT	SYT	Minas Gerais, Brazil
Callicebus nigrifrons	Female	A/S	Y	T	N	AYT	SYT	Minas Gerais, Brazil
Callicebus nigrifrons	Female	A	Y	T	N	AYT	SYT	Minas Gerais, Brazil
Chiropotes albinasus	Female	S	Y	T	N	SYT		Maués Açú River, Amazonas, Brazil
Chiropotes israelita	Female	A	Y/F	T	N	AYT	AFT	Ig Anta, Amazonas, Brazil
Chiropotes israelita	Female	A	F	A	N	AFA		Ig Anta, Amazonas, Brazil
Chiropotes israelita	Female	A	F	T	N	AFT		Marauia River, Amazonas, Brazil
Chiropotes israelita	Male	A	F	A	N	AFA		Demeni River, Amazonas, Brazil
Chiropotes satanas	Male	A	F	A	N	AFA		Unknown locality
Pithecia irrorata	Female	A	F	A	N	AFA		Autazes, Amazonas, Brazil
Pithecia irrorata	Female	A	Y/F	T	N	AYT	AFT	Jari Lake, Amazonas, Brazil
Pithecia pithecia	Male	A	F	T	N	AFT		Manaus, Amazonas, Brazil

3.4 Discussion

The results reported here show that the diversity of M/L opsins found in *Cacajao, Callicebus, Plecturocebus, Cheracebus, Chiropotes,* and *Pithecia* is greater than previously reported. Although the samples' origins and the sample sizes were not appropriate to determinate the opsin frequency, our results contribute to the knowledge of colour vision polymorphism in New World primates. Some allelic variations were found in only one individual per species requiring further studies for confirmation. For instance, the allele SFA was found in one specimen of *Cheracebus lugens*, and we suggest further confirmation.

We found five alleles in black-headed uakaris, the *Cacajao melanocephalus* group (*Cacajao ayresi, C. hosomi,* and *C. melanocephalus*) suggesting a similar diversity to their red-faced counterpart *Cacajao calvus* (Corso et al., 2016; Boubli et al., 2008; Ferrarim, Guedes, Figueiredo-Ready, & Barnett, 2014). Thus, we suggest that the red colourful display found in *C. calvus* is not the ultimate cause for the high allelic variation of the Medium/Long wavelength sensitive opsin in New World primates, thus, challenging the importance of socio-sexual displays in the evolution of routine trichromatic colour vision (Changizi et al., 2006). To the best of our knowledge, this is the first report on the opsin diversity of black-headed uakaris (i.e. *C. ayresi, C. hosomi, C. melanocephalus*).

From physiological studies using electroretinogram flicker photometry, *Plecturocebus* was reported to have the highest number of opsin alleles within a species (*P. moloch*), showing five different M/L photopigments in captive individuals (Jacobs & Deegan, 2005). In a study with wild populations of *P. brunneus* using a molecular approach, three alleles were found representing the most common types from the aforementioned research (i.e. AFA, AFT, and SYT) (Bunce et al., 2011). These three alleles were found for the Callicebinae subfamily in our

analysis. In addition, from the two individuals of *P. moloch* investigated here, the allelic variation SFT was found representing an absorbance peak of 550 λ_{max}nm. Considering the subfamily Callicebinae only, we have evidence of six different alleles, which increases the known opsin diversity found.

In the genus *Chiropotes,* in addition to the alleles AFA, AFT, and SYT reported in the literature (Lima et al., 2015), we found an additional variant AYT with a peak spectral sensitivity of 555 λ_{max}nm in *Chiropotes israelita*, thus, increasing the number of functional alleles from three to four in this genus. Furthermore, the genus *Pithecia* was reported with the alleles AFA, AFT, and SYT (Boissinot et al., 1998). We found a variant AYT allele with the sensitivity peak of 555 λ_{max}nm, increasing the number of known opsin alleles from three to four.

The SYA allele is commonly found in howler monkeys (genus *Alouatta*), and has recently been found in *Callithrix jacchus* (Amador, 2015; Kawamura, 2016; Matsushita et al., 2014). Alleles thought to be present in certain species groups are now being found in different species. Further genetic and ecological studies should indicate if all allelic combinations according to the "three-site rule" are present in New World primate species and how the resulting phenotypes will interact with the environment. For instance, the benefits of trichromatic colour vision for folivory has been widely discussed in the literature (Dominy & Lucas, 2001). Since the only routinely trichromatic colour vision New World primate (i.e. *Alouatta*) feeds mainly on leaves, and the increased opsin diversity in Callitrichines, which mostly forage on insects, fruit and gum, it would be interesting to investigate how similar trichromatic colour vision phenotypes in *Alouatta* and *Callithrix* perform in colour based ecological tasks, thus, helping us to understand the role of highly polymorphic colour vision in New World primates (Araújo et al., 2008; Garber, Estrada, & Bicca-Marques, 2008; Surridge & Mundy, 2002).

Spectral shifts in opsin sensitivity could also result from mutations in other sites of the M/L opsin gene (Shyue et al., 1998). However, the M/L opsin sensitivity peaks are best explained by the "three-site-rule" (Yokoyama & Radlwimmer, 2001). Now that this variation in Pitheciidae is known, one possible approach is to confirm the sensitivity of this protein *in vitro*. cDNA can be used to produce functioning opsins *in vitro* by cloning in cultured cells to measure the photopigment sensitivity by spectrophotometric measurements (Hiramatsu, Radlwimmer, Yokoyama, & Kawamura, 2004). Alternatively, electroretinography from Pitheciidae species in captivity would help to confirm the high allelic variation found.

Similarly to Corso *et al* (2016), we found no evidence of routine trichromatic colour vision in the red-faced uakari. Additionally, we also found no evidence of routine trichromatic colour vision in the black-headed uakaris. If routine trichromatic colour vision was found in the red-faced uakari, as in *Alouatta* (Araújo et al., 2008), this would support the importance of socio-sexual signals in the evolution of colour vision in primates (Changizi et al., 2006), does not seem to be the case. However, both cryptic and conspicuous *Cacajao* morphs share high opsin diversity. This results in an increased number of heterozygotes and potentially more trichromatic females in a group. Similarly in Callicebinae (a subfamily in which most of facial skin is covered with fur) shows high allelic diversity again resulting in a high proportion of trichromatic females.

Despite the fact that trichromatic colour vision is best suited to distinguish colour modulations on the skin (Changizi et al., 2006), there is evidence that the ability to discriminate red colours in primate vision evolved after red visual traits in primate species (Fernandez & Morris, 2007). For example, primate species that are able to discriminate red-green hues have less red fur than dichromate species (Kamilar, Heesy, & Bradley, 2013). Other species, such as *Chiropotes albinasus,* have strong red facial marking, but do not have routine trichromatic colour vision.

Geographical and ecological factors may also affect the morphology of primate species. Group size and incidence of UV light may lead to more complex faces and dark facial masks (Santana et al., 2012). Thus, variations in facial colouration are expected to be generated from both social and biogeographical pressures.

The exaggerated reddish displays in uakaris and the coppery coloration in Callicebinae could be an evolutionary adaptation to allow dichromatic colour vision individuals to identify social signals. Further studies measuring skin and fur colouration would be useful to understand the role of exaggerated reddish displays in New World primates, for instance, if variations in the red-faced uakari are detectable by dichromatic colour vision individuals. This would indicate why these species show this exaggerated colouration pattern. Future research should focus on the benefits of primate groups possessing both dichromatic and trichromatic individuals rather than focusing only on the consequences of different colour vision in an individual primate.

Chapter 4 - Are predator richness and predator type related to colour vision polymorphism in New World primates?

Abstract

Most New World primates feature a polymorphic colour vision system where males have a dichromatic colour vision and females can either have trichromatic or dichromatic vision. The role of such complex visual system can be related to finding food, perception of socio-sexual displays and predator detection. Investigating the predator detection hypothesis in nature is difficult because predatory events are rarely observed. Here we present an investigation of how spatial organisation of New World primate species, on a continental scale, are related to environmental factors and predatory pressures using a biogeographic analysis of primates and their predators. When considering phylogenies (i.e. species as non-statistically independent units), no effect of body size or predator type was found. Snake richness was negatively related to group size, but no effect was found for mammal and raptor richness. Predator richness did not significantly affect the distribution of primate species with monochromatic or trichromatic vision, while polymorphic colour vision primate species were affected by predator type. We conclude that the lack of effect of predator species richness found along the trichromatic colour vision species distribution indicates an adaptation to predator detection.

4.1 Introduction

New World primates possess a polymorphic colour vision system where males have dichromatic colour vision, similar to colour blind humans, and females can either have trichromatic or dichromatic colour vision (De Valois & Jacobs, 1968; Jacobs, 1996; Jacobs, 2007; Jacobs & Rowe, 2004). Thus, in most species of New World primates groups are composed of both trichromatic and dichromatic individuals. This polymorphic situation is caused by a sex-linked gene for the medium-long wavelength sensitive opsin (i.e., protein responsible for converting light into electrochemical signals for the brain) located on the X (sex) chromosome (Dulai, von Dornum, Mollon, & Hunt, 1999). The genus *Aotus* have a monochromatic colour vision, not being able to differentiate colours, probably, as a result of its nocturnal behaviour (Donati & Borgognini-Tarli, 2006; Jacobs, Deegan, et al., 1993), Whereas the genus *Alouatta*, has duplicated the gene in the X chromosome, as the Old World primates have done, resulting in trichromatic colour vision (Araújo et al., 2008; Julliot & Sabatier, 1993).

The evolutionary explanation for polymorphic colour vision in primates is still unclear in the scientific literature: does polymorphism in colour vision has some fitness consequences (Fedigan, Melin, Addicott, & Kawamura, 2014; Jacobs & Bradley, 2016)? However, several hypotheses have been proposed relating to foraging, social, or predation pressures. Trichromatic individuals are better adapted to detect colour variations in food items and more able to identify ripe fruits and edible leaves (Lucas et al., 2003; Osorio & Vorobyev, 1996; Regan et al., 2001), while dichromatic colour vision individuals have a better vision in low light levels and can better detect camouflaged stimuli (Caine et al., 2010; Morgan et al., 1992). The balance between these two features might be responsible for the maintenance of the

polymorphic colour vision system found in these species (Riba-Hernández et al., 2004), especially since New World primate groups will often be composed of trichromatic and dichromatic individuals.

Another potential advantage of the polymorphic colour vision system is predator detection. Theoretically, cryptic predators, such as snakes, would be better detected by dichromats (Saito et al., 2005), while trichromatic individuals would be better able to detect conspicuous predators (Pessoa et al., 2014). Researchers have demonstrated the performance of both colour vision systems in detecting predator threats. For example, Pessoa and collaborators (2014), have shown that normal colour vision humans (trichromats) outperform colour blind individuals (dichromats) in detecting carnivore predators in a naturalistic setup. However, testing predator detection by different colour vision phenotypes is difficult to accomplish in the wild as predation events are rarely observed. One possible alternative is to investigate the occurrence of traits present in areas with different predatory pressures.

The predators of New World primates are either raptors, mammalian carnivores or snakes (Ferrari, 2008). Raptors are abundant and have predation strategies ranging from aerial searching, ambush and long chase flights to attack their prey (Hart, 2007; Robinson, 1994). Several feline species have been identified as mammalian primate predators (Hart, 2007). Predation by large snake species, such as *Boa constrictor* and venomous snakes, such as rattlesnakes represent a significant threat (Boinski, 1988; Cisneros-Heredia, León-Reyes, & Seger, 2005; Corrêa & Coutinho, 1997). The geographic distribution of both primates and predators generates different selective pressures in different geographic areas, and therefore allows the testing of favoured traits (Thompson, 1999). Group size and body size has an established relationship to predation pressure, with predation risk declines with the increase in group size or body weight (Isbell, 2005). The behavioural strategy to avoid predation is

equally informative such as visual detection by many eyes (Lima, 1995; Roberts, 1996). In this sense, the sensory abilities are relevant; for example, if the many eyes have different perceptual capabilities and are coopertative then predator detection could be more efficient (Morgan et al., 1992; Pessoa et al., 2014; Surridge et al., 2005).

Here we investigate the spatial organisation of Neotropical primates and their predators verifying the variation of species' richness geographically and then determining which variables best predict primate distribution (such as environmental variables or predators). We predict that environmental variables will partially explain the distribution of primates; the established relation of predator risk and body size or group size will be observable in geographic scale.For instance, small primates should be more succeptible to raptor predation while heavier primates will be more affected by mammalian predation. Colour vision systems will be affected by predator richness allowing a conclusion concerning the role of vision in New World primate species (e.g. best suitable phenotypes, such as routine thrichormatic colour vision, will not be affected negatively by predator richness). Our main aim is to analyse the effects of predator richness and predator type on the occurrence of polymorphic colour vision traits in New World primates.

4.2 Methods

To investigate the effect of predator richness on primate colour vision traits a grid of 1350 cells of measuring 1x1 decimal degree, was built over the full platyrrhine distribution using ArcGIS 10.3 and the Geospatial Modelling Environment extension (Beyer, 2015; ESRI, 2008) (Figure 6). This selected area was then used to define the richness of co-occurring predator-prey species. A total of 142 primate species distribution polygons from 19 platyrrhine genera were used to calculate the primate richness in number of species in each grid cell (IUCN 2015). Bird predator species were identified from the scientific literature concerning recorded predation events on New World primates and 18 species of raptors were included as reported avian predators (del Hoyo & Elliott 1994; Hart 2007; BirdLife International and NatureServe 2013). Similarly, mammalian predators, including felines and mustelids, from reported cases in the scientific literature, were identified and are listed in the Appendix I (Bianchi & Mendes 2007; Hart 2007; Ferrari 2008; IUCN 2015). Constrictors and venomous snakes families were all selected due to the strong response they evoke in primates (Boinski, 1988; Hart, 2007; Isbell, 2006). A total of 162 genera from Colubridae, Elapidae, Boidae and Viperidae were included in the analysis (GBIF, 2015). Point data were used to create minimum convex polygons for snake distributions using GME extension for ArcGIS 10.3 (Beyer, 2015; ESRI, 2008). Capuchin monkeys (*Cebus sp*.) and other exceptional cases of predator species such as coatis (*Nasua sp.*), toucans (*Ramphastos sp.*), and caimans (*Caiman sp.*) despite occasional reports were not included as predators (Boinski, 1987; Ferrari, 2008; Rose et al., 2003; Sampaio & Ferrari, 2005). Species distribution polygons of mammalian and avian predators were obtained from the International Union for Conservation of Nature (IUCN 2014) and NatureServe (2013). Predator distributions were compared between each other using the

package ecespa using Cramer-von Mises test (Masetre, Escudero, & Bonet, 2010). This analysis allowed to compare predator distributions to find if there are significant spatial differences between two distributions.

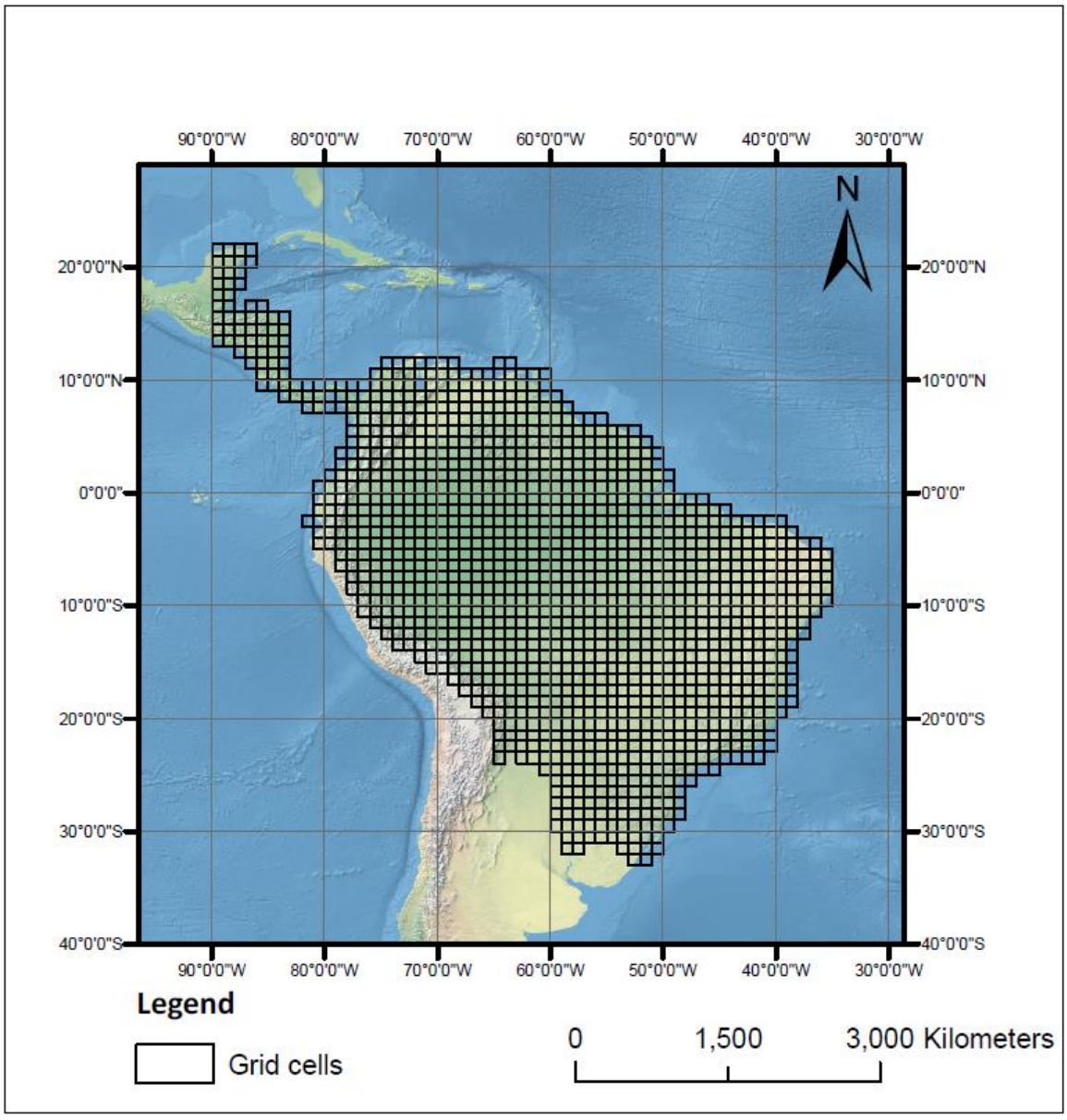


Figure 5: Grid cells based on primate species distribution

Environmental variables considered were mean annual temperature in degrees Celsius, mean annual precipitation in millimetres, altitude in metres, and Global Potential Evapo-Transpiration (PET) (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005; Trabucco & Zomer, 2009). Temperature and precipitation were obtained from annual mean from 1950 to 2000 with a resolution of 30 arc-seconds (approximately one kilometre); altitudinal data followed the same resolution (Hijmans et al., 2005). The PET data demonstrated a potential for vegetative growth at a resolution of 30 arc-seconds (the annual mean from 1950-2000) (Trabucco & Zomer, 2009) (Figure 7).

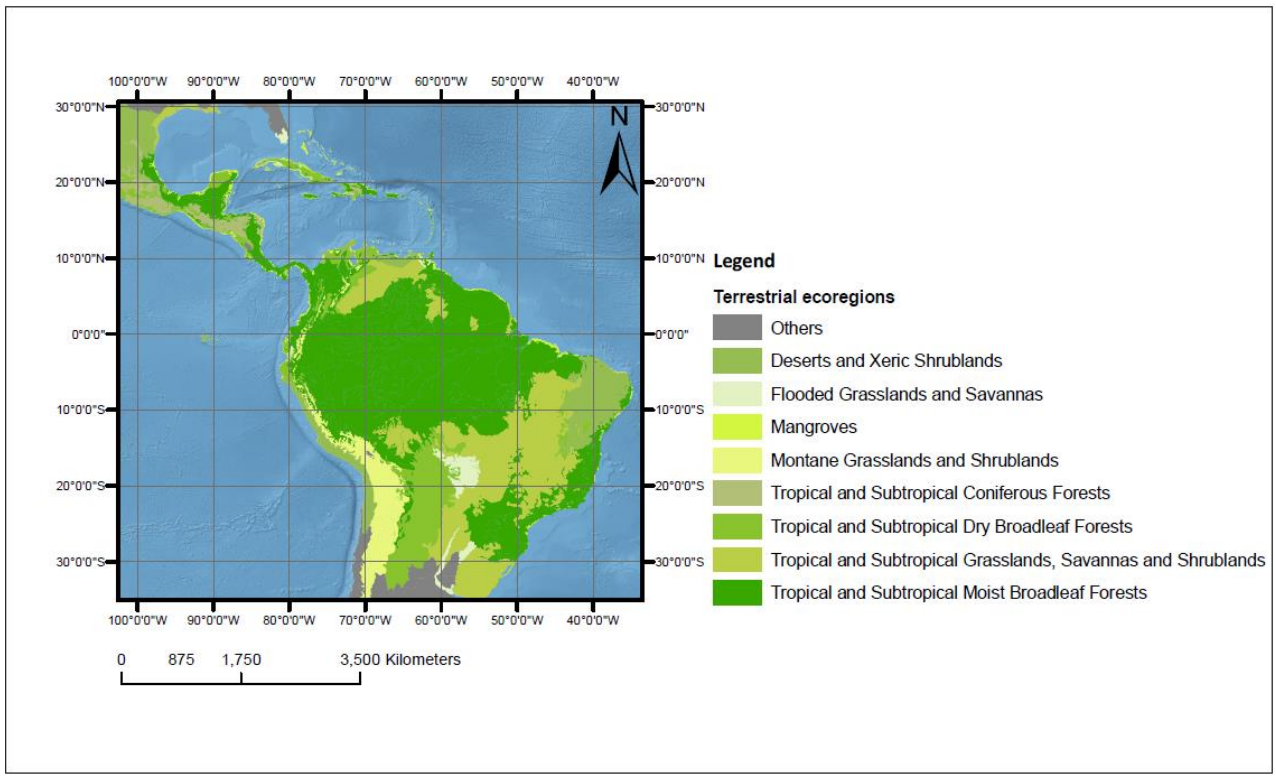


Figure 6: Terrestrial ecoregions in Central/South America

A matrix of species by spatial unit (1 x 1 decimal degrees grid) was overlapped with polygons of species' range to find the number of species per cell and the related environmental data. The data were checked for Spatial Autocorrelation (Moran's I) using the Spatial Statistics

module in ArcGIS 10.3 (ESRI, 2008) and SAM 4.0 (Rangel, Diniz-Filho, & Bini, 2010). The Moran's I Index is a spatial autocorrelation analysis, which evaluates if occurrences are distributed evenly or clustered in space (Chen, 2013; Moran, 1948). The existence of spatial autocorrelation means that the observed data were not independent (i.e. geographically biassed). A Geographically Weighted Regression (GWR) and an Ordinary Least Squares Regression were performed to understand the pattern of distribution of primate species if the data presented a Spatial Autocorrelation using ARCGIS 10.3 (ESRI, 2008).

To verify the effect of group and body size, New World primate genera were grouped into two major categories. Primate body size classes were grouped according to body weight in the following categories: Miniature (<0.7 kg) including *Callibella, Callimico, Callithrix, Cebuella, Leontopithecus, Mico,* and *Saguinus*; Small (0.7-2 kg) with *Callicebus,* and *Saimiri*; Medium (2-5 kg) with *Cacajao, Cebus, Chiropotes* and *Pithecia*; Large (>5 kg) *Ateles, Brachyteles, Lagothrix* and *Oreonax* (Ferrari, 2008). Group size classes were grouped according to social organization and number of individuals as follows: Nuclear family including *Callicebus* usually ranging from two to seven group members; Extended family including *Callibella, Callimico, Callithrix, Cebuella, Leontopithecus, Mico, Saguinus,* and *Pithecia* ranging from four to 20 group members; Harem including *Cebus* also with four to 20 group members; and Large including *Ateles, Brachyteles, Cacajao, Chiropotes, Lagothrix, Oreonax,* and *Saimiri* showing more than 20 individuals in the groups. A phylogenetic generalised linear mixed model was performed to verify the effect predator richness on the distribution of primates varying in body size and group size. This approach was used to control for the phylogenetic signal on primate distribution (Hadfield, 2010). The phylogeny used was based on Perelman and collaborators (2011) (Figure 8).

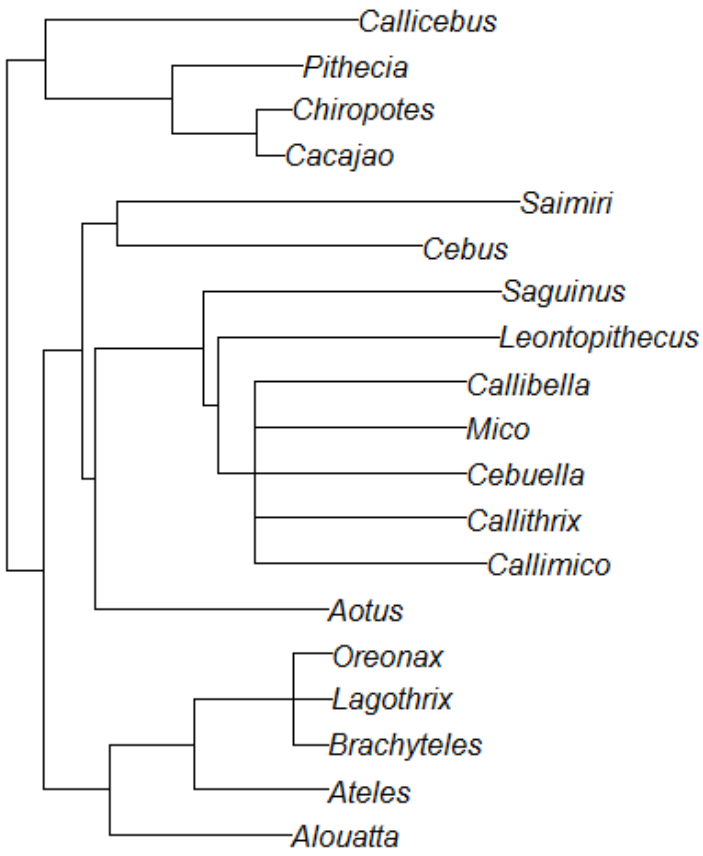


Figure 7: Phylogenetic tree considered to perform statistical analysis correcting species as non-independent statistical units.

To investigate the effect of polymorphic colour vision on primate distribution, we used the occurrence of each primate species as response variable and the colour vision type (i.e., monochromat, dichromat, and trichromat) nested by predator type (i.e. mammals, snakes, and raptors) corrected for phylogeny using a phylogenetic generalised linear model (Hadfield, 2010)(Hadfield, 2010)(Hadfield, 2010)(Hadfield, 2010). The GLMMs were performed using the package MCMCglmm and RStudio (Computing, R Foundation for Statistical Vienna, 2015; Hadfield, 2010; RStudio Team, 2015).

4.3 Results

4.3.1 Patterns of distribution

Mammal predators were present in most grid cells with a mean species' richness of 6.45±1.19 and were only not present in eight of 1350 grid cells (Figure 9). The mean raptor species' richness per cell was 10.6±2.80 and only 4 grid cells did not show any avian predators (Figure 10). Snake species' richness per cell was 42.9±10.93 and only four grid cells had no snake species (Figure 11). When comparing predator distributions Mammals with Raptors (psi=0.5271, P=0.0001), Mammals with Snakes (psi=2.1103, P=0.0001), and Snakes with Raptors (psi=1.0086, P=0.0001) they presented distinct distributions across the New World primate species' ranges (Fig. 1).

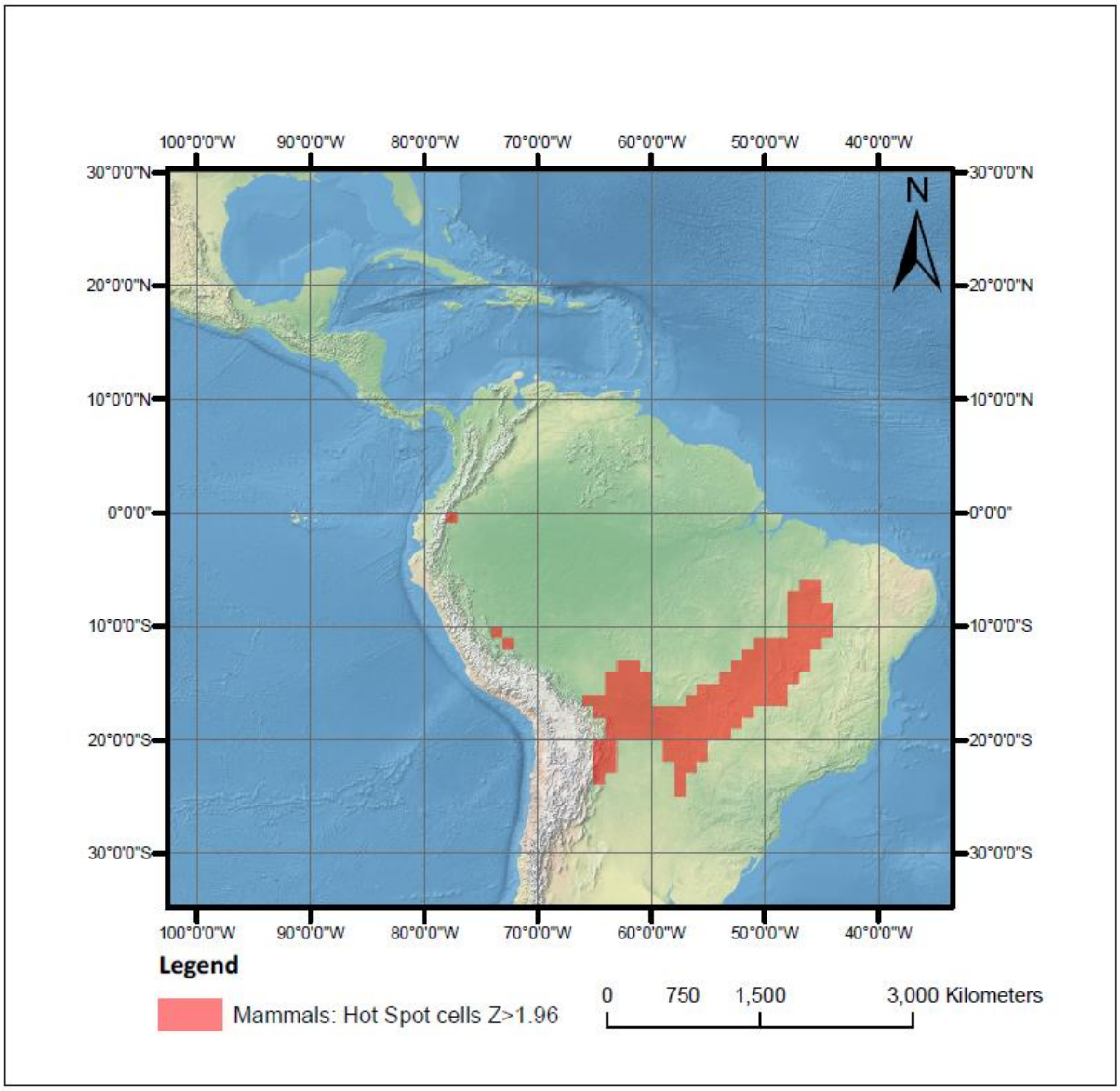


Figure 8: Distribution of Hot Spot grid cells of mammal predators

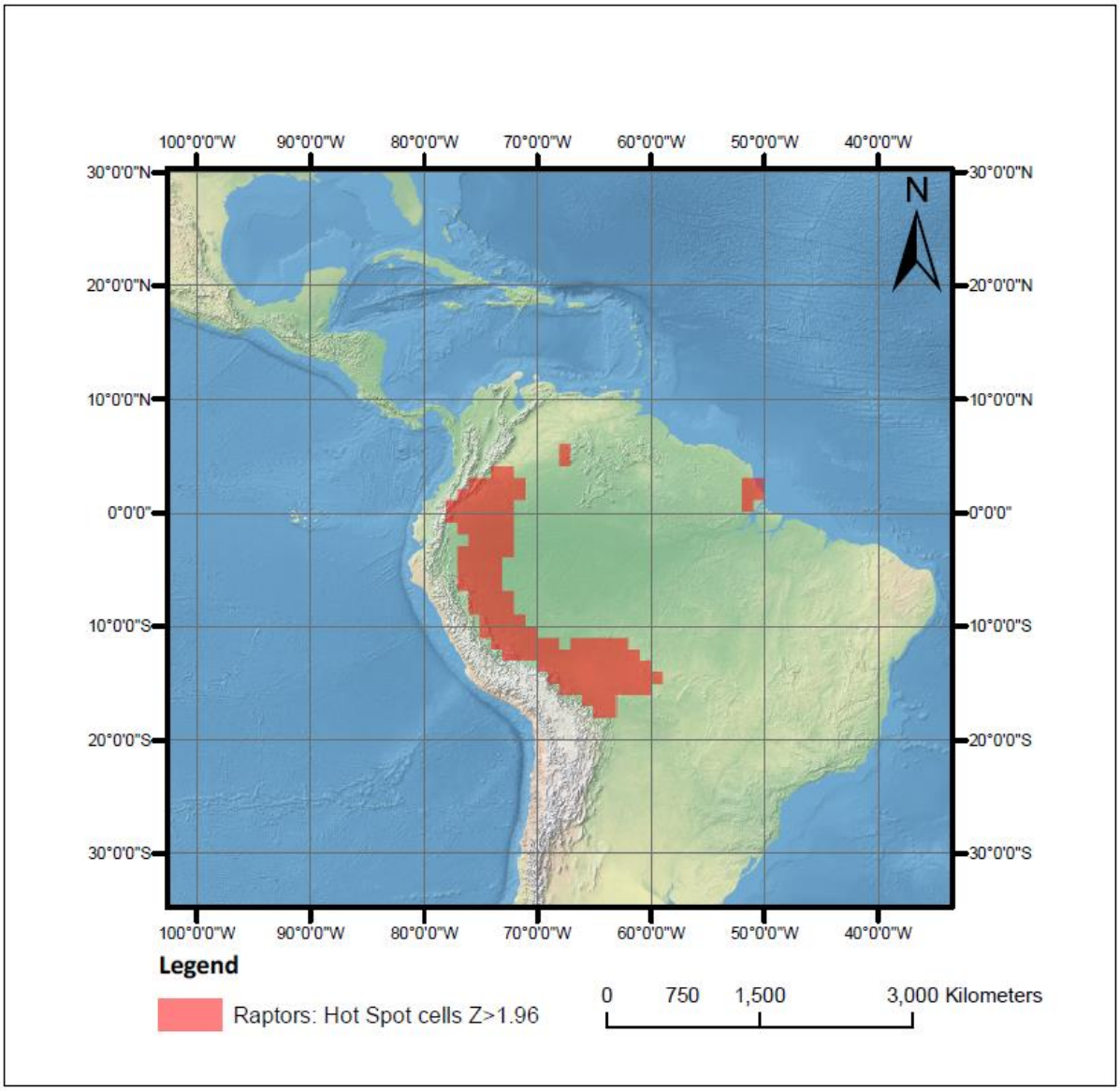


Figure 9: Distribution of Hot Spot grid cells of Bird predators

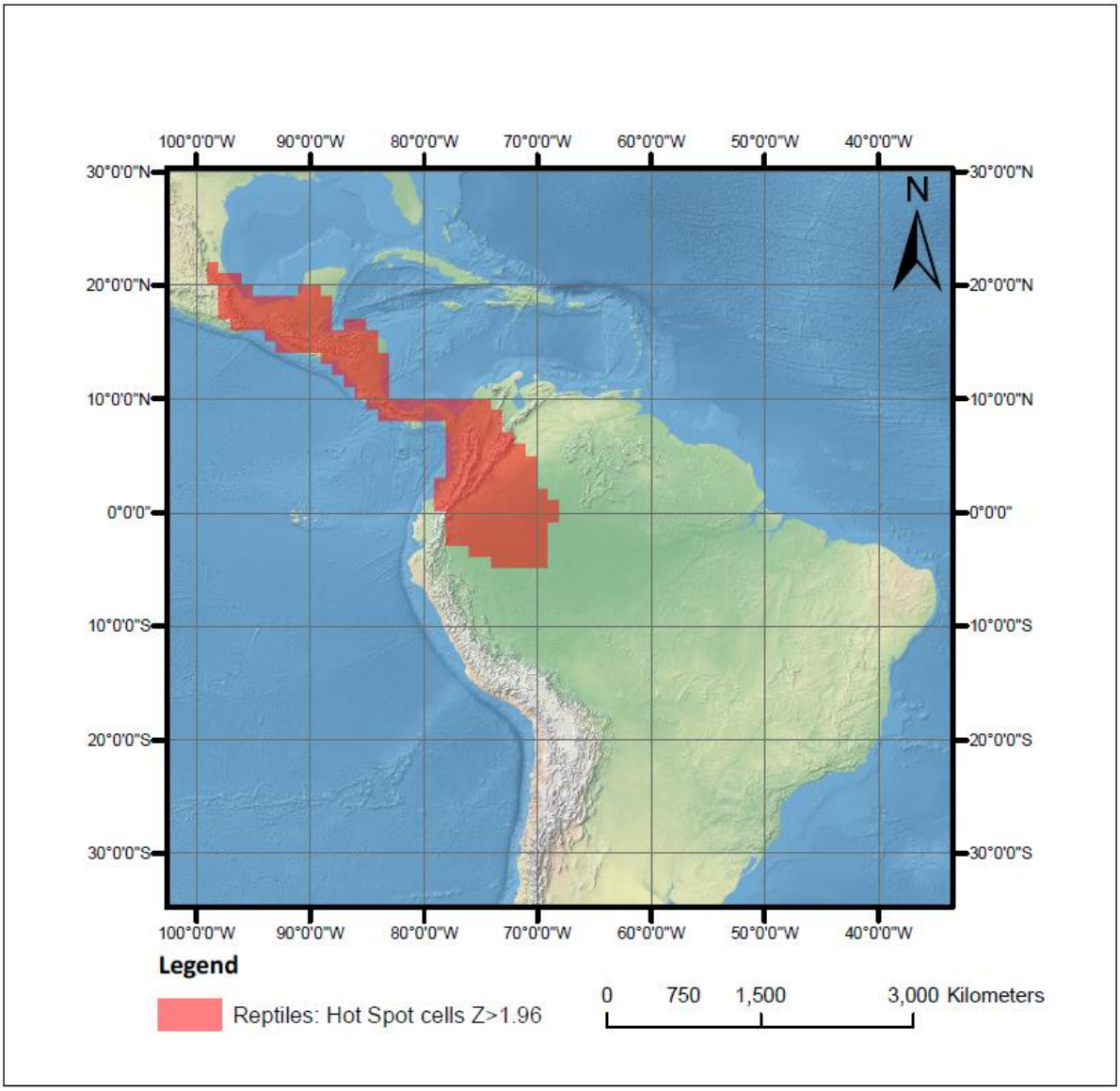


Figure 10: Distribution of Hot Spot grid cells of Reptile predators

All primate species and predators studied showed a global spatial autocorrelation. Snakes (Moran's I=0.87; P<0.05), raptors (Moran's I=0.68; P<0.05) and mammals (Moran's I=0.61; P<0.05) did not have a random distribution in space. The same was observed for *Aotus* (Moran's I=0.82; P<0.05), *Alouatta* (Moran's I=0.68; P<0.05), Miniature sized primates (Moran's I=0.87; P<0.05), Small (Moran's I=0.86; P<0.05), Medium (Moran's I=0.85; P<0.05), and Large primates (Moran's I=0.85; P<0.05). Nuclear family groups (i.e. *Callicebus*) (Moras's I=0.76; P<0.05), extended family groups (Moran's I=0.88; P<0.05), harems (*Cebus*) (Moran's I=0.74; P<0.05), and large groups (Moran's I=0.87; P<0.05), also showed spatial

autocorrelations, meaning that these distributions should undergo statistical testing that considers spatial autocorrelation (Figure 12).

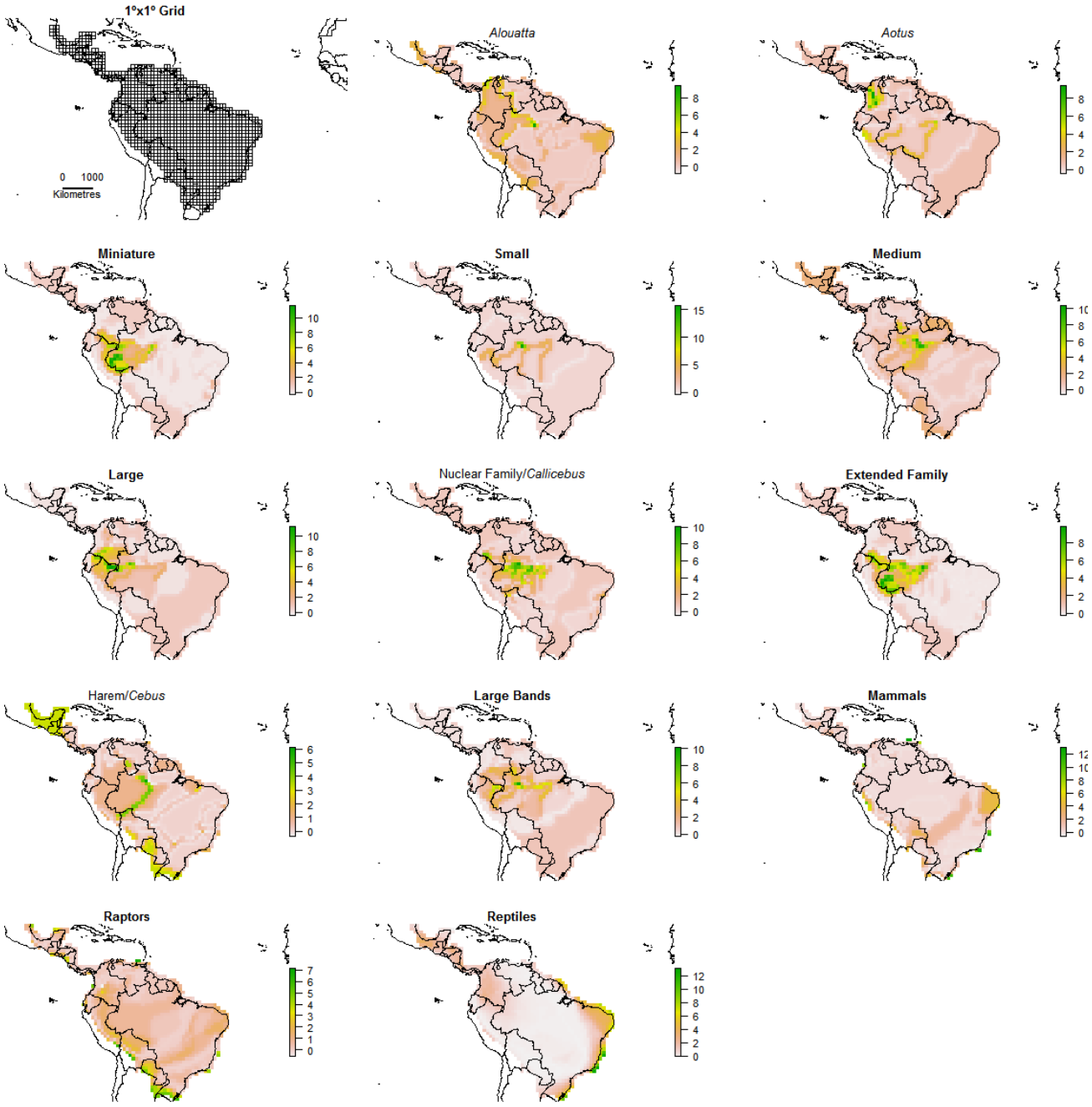


Figure 11: Moran's I global spatial autocorrelation of predator type, primate body size, group size and genera.

The occurrence of primates was predicted by environmental variables through GWR with PET, precipitation, temperature, and altitude responsible for 87% of primate distribution (R^2=0.871, F (1255.71) = 56.5294, P<0.05). All explanatory variables, PET (β=0.0306, P<0.01),

precipitation (β=0.0.003, P<0.01), temperature (β=0.4095, P<0.01), and altitude (β=0.0014, P<0.01) had a positive relationship towards primate distribution.

4.3.2 Phylogenetic analysis

The mean phylogenetic signal in the sample was 68.83% (λ=0.6884), with a posterior mode of 0.9187, and the 95% credible interval λ ranging from 0.1423-0.998.

Body weight was not a significant factor explaining the relationship of primate and predator richness. We found no significant coefficients for body weight and the explanatory variables mammals (β=-68.984, $CI_{lower/upper}$=-758.632/304.474, P=0.870), raptors (β=4.981, $CI_{lower/upper}$=-117.705/124.741, P=0.900), and snakes (β=2.463, $CI_{lower/upper}$=-12.836/18.224, P=0.813). Regarding group size, mammals (β=-151.746, $CI_{lower/upper}$=-411.928/683.602, P=0.5933) and raptors (β=121.841, $CI_{lower/upper}$=-69.790/317.606, P=0.27) had no effect on group composition; however, snakes (β=-28.330, $CI_{lower/upper}$=-57.997/-2.021, P=0.0433) had a negative effect on primate group size with species with a smaller group size occurring less where the snake richness was high.

Predator types nested by colour vision types show that monochromatic colour vision is not significantly predicted by mammal (β=-744.00, $CI_{lower/upper}$=-191100/195100, P=0.98), raptor (β=-444.30, $CI_{lower/upper}$=-196900/181800, P=1) or snake richness (β=200.50, $CI_{lower/upper}$=-49810/51940, P=0.99). We observed no significant effect in trichromatic colour vision species predicted by mammals (β=667.10, $CI_{lower/upper}$=-193300/194700, P=0.99), raptors (β=353.20, $CI_{lower/upper}$=-200200/182200, P=0.99) or snakes (β=-187.30, $CI_{lower/upper}$=-54380/58270, P=0.98). However, mammal richness had a positive effect on the occurrence of polymorphic colour vision species (β=0.34, $CI_{lower/upper}$=0.02/0.68, P=0.04), a negative effect with raptors

(β=-0.26, $CI_{lower/upper}$=-0.49/-0.03, P=0.03), but no effect from snake richness (β=-187.30, $CI_{lower/upper}$=-54380/58270, P=0.06).

4.4 Discussion

Different colour vision systems (monochromatic, dichromatic, and trichromatic) have been regarded as having different abilities to detect predators. While they present divergent results in experiments of predator detection (Morgan et al., 1992; Pessoa et al., 2014), we have here presented indirect evidence of trichromatic colour vision as the most appropriate phenotype for predator detection by analysing the richness patterns in predator and prey species. First, in spite of all primate predators being distributed across all Platyrrhini distribution, we found that mammals, snakes, and raptors present distinct richness patterns. In predator rich areas, primate populations would be under stronger selective pressure. As predator types had a significant difference in distribution, it is possible to understand the effect of each type of predator in the colour vision system. This does not mean that predators were exclusive in some areas, but the difference in species frequency between predator types showed how varied the selective pressure was. For instance, an area rich in raptors and the same time rich in mammalian predators have a negative and positive effect respectively in polymorphic colour vision primates. This is relevant to understanding how colour vision traits or polymorphisms are related to different predation pressures.

As expected, environmental variables are useful for predicting primate richness. By using the occurrence of primate species in grid cells we were able to investigate the factors that affect primate distribution. Since, predators were not evenly distributed across primate species range, this poses the question: What are the effects of predator richness in primate distribution? We found no relationship with predator type and primate body size. Ferrari (2008) states that larger mammals are more prone to be predated by mammals, while raptors and snakes are a more significant threat to small-bodied primates. However, we did not find

this pattern on a landscape scale. Indeed, large-bodied primates are threated mostly by large mammal species because raptors have a limit to the weight of prey they can carry. However, this does not exclude the predation of smaller primate species, predation of infants and juveniles, or predation of primate species up to 6 kilograms by raptors. Most New World primates weight less than five kilogrammes (Mittermeier et al., 2013). Even the largest raptor species in the New World, *Harpia harpyja*, preys upon primates up to 6 kilograms and most other species preying often lighter prey (Ferrari, 2008).

Primate group size had a negative relationship only with snakes. For instance, Venomous snakes can endanger a primate without predating them (e.g., feeling threaded and biting by defense). Larger group sizes were present in areas with lower snake richness. Snakes are primate predators, but they are also a physical threat. While mammals and raptors are active search predators, snakes are mostly ambush predators (Ferrari, 2008; Hart, 2007; Thiollay, 1985). Group size is often related to vigilance in primate groups. Usually, individuals in larger groups are less vigilant than in smaller groups (De Ruiter, 1986). However, it is known that group size does not decrease the individual's risk of predation when predators do not rely on surprise (Treves, 2000). It is predicted that smaller groups will have fewer encounters with snakes when compared to a larger group. Due to competition among individuals, the daily range increase with the group size increase, which we assume that with a wider territory cover by primate species there will be more opportunities to face an ambush predator, such as snakes (Wrangham, Gittleman, & Chapman, 1993). Nevertheless, group size and body size did not fully explain the patterns of distribution of New World primates in this study.

Body weight and group size are considered as two principle features determining predation risk (Isbell, 2005). However, behavioural strategies also play an important role in predation avoidance. For instance, primates can mob snakes during encounters (Boinski, 1988; Tello,

Huck, & Heymann, 2002; Vitale, Visalberghi, & De Lillo, 1991) or employ alarm calls to alert conspecifics, thereby avoiding the encounter (Cäsar et al., 2012, 2013). Thus, the ability to detect the predator is especially relevant to the predator-prey interaction and coevolution. While body weight limits prey capture, group size rely on the detection ability to provide an appropriate behavioural response. As vision is the predominant sense for predator detection in primates, the effect of group size is dependent on the detection ability.

By analysing the colour vision system it is possible to further explore the patterns of primate distribution. Monochromatic species (i.e. *Aotus*) showed no effect for any predator type, possibly, due to it having nocturnal habits to avoid predators (Colquhoun, 2006; Curtis & Rasmussen, 2006). Polymorphic colour vision primate species were positively related by the increase in richness of mammal predators, while raptor species richness had a negative effect. Mammalian predators are an important threat to primates; however, raptors are much more abundant and might represent a bigger threat (Hart, 2007). In the Neotropics, predation by raptor species are the most reported incidences; in Africa predation reports by felids and raptors equally represented; and, in Asia, most reports by mammals (Hart, 2007). The positive mammal richness relationship detected might be related to environmental factors due to a similar physiology. Trichromatic colour vision species (i.e., *Alouatta*), showed no negative effect with predator richness increase (Araújo et al., 2008). This implies a better ability to detect predators in species with routine trichromacy. In summary, monochromatic primates avoid predation by having nocturnal behaviour showing no effect; polymorphic colour vision primates adapted their species distribution being influenced by predator richness in the area (effect not observed in monochromatic and trichromatic colour vision species). ; trichromatic colour vision primate species had no effect as their colour vision system is well suited for early predator detection.

While the evidence here points towards a better performance in detecting predators by trichromatic colour vision individuals, dichromatic colour vision would be better suited to identifying camouflaged insects, as a food source (Caine et al., 2010; Melin et al., 2007; Vogel et al., 2007), increasing the fitness of this individuals and favouring the polymorphism. It would be possible to use the same method employed in the present study to analyse predation on camouflaged insect species by primates. For instance, the routinely trichromatic genus *Alouatta*, feeds mainly on plant species (Cristóbal-Azkarate & Arroyo-Rodríguez, 2007; Julliot & Sabatier, 1993; Pavelka & Knopff, 2004) and would not have an advantage in identifying camouflaged insects for consumption. In contrast, the genus *Brachyteles*, do not have insects as a significant food source and retain a polymorphic colour vision (Strier, 1991; Talebi, Bastos, & Lee, 2005; Talebi, Pope, Vogel, Neitz, & Dominy, 2006). However, the large body size of this species is the result of both its diet (mainly leaves) (Fleagle, 2013) and decreased predator vulnerability – separating out the effects of these two factors would be difficult.

Another major item in New World primates' diet is tree exudate, which is a highly nutritious food source that also might attract insects (Coimbra-Filho & Mittermeier, 1976). Exudate is a significant item for marmosets and its importance increases in periods of shortage in other diet items, such as fruits and insects (Nash, 1986; Rylands, 1984). Further studies could investigate if there is any advantage of dichromatic colour vision in facilitating return to recently gouged trees and eating insects attracted by the sap. Polymorphic colour vision could help to balance the fitness consequences of different genotypes by mediating a primate's performance in finding different food items under conditions of variable food availability.

In conclusion, we show strong albeit indirect evidence of the importance of trichromatic colour vision in detecting predators by showing no effect of predator richness on the

distribution of either routinely trichromatic colour vision (i.e. *Alouatta*) or monochromatic (i.e. *Aotus*) primate species in the New World.

Chapter 5 - Importance of colours for primate predator identification

Abstract

The perception and description of colour camouflaged targets is subjective. Most classification of cryptic and conspicuous animal patters is based on human perception and does not consider other primate sensory abilities. By using computer vision and machine learning it is possible to investigate what information colour camouflaged animals emit and the detectability of this information without human induced bias. Consequently, this method can be used to test the hypothesis concerning which colour vision phenotype (trichromat or dichromat) can best use the colour information present in digital images to detect predators. Here we compare the accuracy of models trained with colour blind simulation images, full colour images, and an achromatic computer vision model to investigate the detectable predators were by different colour vision phenotypes. We found that all models were able to distinguish the control group from predators. However, the dichromatic colour vision computer models had the lowest performance when compared to the achromatic and full colour models. The low accuracy of dichromatic models challenges the traditional assumption of dichromatic colour vision being more suitable to breakthrough camouflage. Future research should investigate the interaction of the importance of light levels and the interaction of colour and achromatic contrast for predator detection and recognition.

5.1 Introduction

Using biology to inspire design is a common approach in engineering. For instance, this is used in equipment design, such as in bio-inspired robot locomotion (Hoover, Burden, Xiao-Yu Fu, Shankar Sastry, & Fearing, 2010); using penguin hunting behaviour to develop an algorithm for safe breaking systems in cars (Gheraibia et al., 2015; Helms, Vattam, & Goel, 2009; Tero et al., 2010); and structure design, such as biologically inspired by geckos' feet to design climbing equipment (Liu & Jiang, 2011). It is also possible to use engineering or simulation approaches to test hypotheses in biology. Many biological problems are difficult to investigate in the wild or in the laboratory due to logistical, ethical and measurement limitations: one solution to this problem is to use a simulation or engineering approach (Mariti et al., 2015; Webb & Consi, 2001). Biorobotics can employ robots as physical models of biological systems to test biological hypothesis, such as the impact of personality types in the study of social behaviour (Beer, Chiel, Quinn, & Ritzmann, 1998; Krause, Winfield & Deneubourg, 2011; Webb, 2000). Similarly, computer neural networks can be used to test ecological hypothesis, such as the investigation of the confusion effect in predator avoidance (Tosh, Jackson, & Ruxton, 2007).

Much colour vision research is done by modelling mathematically the perception abilities of animals. Due to the relationship between genotype and the photopigment phenotype (i.e. opsin and rhodopsin) it is possible to predict accurately the threshold of spectral sensitivity and colour discrimination abilities in species (Vorobyev & Osorio, 1998). This provides a quantitative measurement allowing hypothesis testing and fitting of behavioural data thereby revealing neural mechanisms constraining behavioural judgments (Kelber et al., 2003). For

example, studies of visual communication by colourful displays in birds, primates and insects use this approach to evaluate signal detection (Osorio & Vorobyev, 2008).

Dichromatic colour vision is the most common phenotype among mammals (Bowmaker, 2008). Only a few groups, such as Old World primates and some marsupial species feature trichromatic colour vision (Arrese et al., 2002, 2006; Rowe, 2002). Reptiles, fish and birds have trichromatic or tetrachromatic colour vision, thereby being able to detect much more colour variation (Bowmaker, 2008). Thus, it is common among mammals to have vision similar to a colour-blind human (Jacobs, 1990). Dichromatism in primates is an evolutionary consequence of a nocturnal mammalian ancestor, with trichromatic colour vision being recently acquired in primate groups (Bowmaker, 1998; Hunt et al., 1998). While Old World primates have a routine trichromatic colour vision, New World primates retain both phenotypes within a population (Dulai et al., 1999; Jacobs & Deegan, 1999).

By identifying the number and types of photoreceptors in primate species, it is possible to reveal the complexity of colour vision in New World primates. These species feature polymorphic colour vision in which males are obligatory red-green colour blind (i.e. dichromatic, except for howler monkeys) and females can present either a dichromatic or trichromatic colour vision (De Valois & Jacobs, 1968; Jacobs, 2007; Mollon et al., 1984). The adaptive role of such polymorphism is still debated; however, it is known that trichromatic colour vision individuals have advantages in identifying ripe fruit and new leaves, whereas dichromatic colour vision individuals have vision better adapted to low light levels and to detect camouflaged objects, such as insects (Dominy & Lucas, 2001; Matsumoto et al., 2014; Melin et al., 2007; Morgan et al., 1992; Regan et al., 2001; Saito et al., 2005; Verhulst & Maes, 1998).

Knowledge concerning the diversity of photoreceptors in New World primates is increasing. Among these primates, some species show a high number of polymorphisms in the same population due to great opsin allelic variation (Bunce et al., 2011; Corso et al., 2016; Jacobs & Deegan, 2005). As a result, the number of trichromatic colour vision females increases. However, the number of anomalous trichromatic colour vision females (i.e., reduced red-green discrimination) also increases. Howler monkeys, the only routine trichromatic colour vision primate species in the New World, have been reported with an increased number of anomalous colour vision individuals when compared to Old World primates (Araújo et al., 2008; Matsushita et al., 2014). Therefore, the role of defective colour vision and dichromatic individuals in a population needs to be further investigated.

Predator identification and recognition is extremely important in primate behaviour. Primates are able to use referential communication to perform an appropriate behavioural response and avoid predation (Cäsar et al., 2012, 2013; Cheney & Seyfarth, 1990). This means, if a bird predator is detected by one and communicate with the group, they will respond by sheltering below the canopy, while if a mammal predator is detected the group will avoid the ground substrate. Dichromatic colour vision individuals should be more able to detect camouflaged predators; however, the performance of trichromatic colour vision individuals was shown to be better than dichromats under naturalistic conditions (Pessoa et al., 2014). In fact, selection for the detection of snakes is one strong hypothesis for the evolution of trichromatic colour vision in primates (Isbell, 2006). Thus, the role of dichromatism for predator detection needs to be further investigated.

Machine learning is the process where the computer learns a task using a training set with examples: in the next stage, the same task is performed using new data (Louridas & Ebert, 2016). In computer vison algorithms, images can be reduced to a series of descriptors that

are a series of compact representations of the images used together to describe and categorize an image (Nowak, Jurie, & Triggs, 2006). Combining these approaches it is possible to create an image classification images system with an accurate performance (Nowak et al., 2006; O'Hara & Draper, 2011).

Computer and animal vision share the same goal, which is to gather and interpret information, and biological vision is a source of inspiration for algorithm design (Kruger et al., 2013; Medathati, Neumann, Masson, & Kornprobst, 2016). With the development of better computing capabilities and the detailing of the neural processes a number of computational techniques are becoming available to achieve bio-inspired computer vision, such as Convolutinal Neural Networks (Medathati et al., 2016; Merolla et al., 2014). It is possible to use machine learning to build automatic classifiers, rather than manual classification (Domingos & Pedro, 2012). For example, using artificial neural networks, computers have been trained to identify a variety of animal species images based only on physical features of the images such as geometry, morphology and texture (Amir, Zahri, Yaakob, & Ahmad, 2017; Hernández-Serna & Jiménez-Segura, 2014; Kumar, Manohar, & Chethan, 2015).

There are two distinct visual pathways in the primate brain prior to cortical processing. The parvocellular pathway processes mainly colour, while the magnocellular pathway is responsive to contrast (Barton, 1996; Livingstone & Hubel, 1988). Associating these pathways and trained models, we compare the parvocellular pathway as the colour based classification model, and the magnocellular pathway as the shape-based classification model. Here we make a parallel between computer vision and primate vision to investigate the importance of colour and shape in the detection of predators by primates with either trichromatic or dichromatic colour vision. This simulation approach does not represent the animal's biology, but reveals the importance of using only colour to correctly classify animals as predators.

Thus, it allows the comparison of the performance of different vision phenotypes in predator detection.

5.2 Methods

5.2.1 Model training

We created three image classifiers. A trichromatic colour vision model trained with a full colour image dataset; a dichromatic colour vision model trained with a colour blind simulation images dataset; and a shape based model using corners, blobs and junctions as interest points (Bay, Tuytelaars, & Van Gool, 2006). Once having trained the models, we tested the classifier with a dataset of camouflaged targets.

To extract information from images, we employed a bag of features technique from the Computer Vision System Toolbox (The MathWorks Inc., 2016), which is a computer vision approach that uses a random collection of image features lacking structure and spatial information (O'Hara & Draper, 2011). A correct classification, selected by the Classification Learner app in Matlab, is assigned when the test image share the highest number of features with the learnt category (Grzeszick, Rothacker, & Fink, 2013). Each image category will have a great number of descriptors and using machine learning is possible to train a classifier to attribute the correct category to the test image. By comparing different classifiers using the Classification Learner app from the Statistics and Machine Learning Toolbox (The MathWorks Inc., 2016), we selected the classification method with the highest accuracy. For instance, with the Decision Trees classifier nodes are created as labels ramifying in branches classifying images based on splitting the data in the subsequent path (Quinlan, 1986); the Discriminant Analysis is a classifier that reduces the number of features to select the best predictor for categories (Mika, Ratsch, Weston, Scholkopf, & Mullers, 1999); the Support Vector Machines works by plotting data in space and finding gaps between different categories, then comparing the nearest category where the new data comes from (Cortes & Vapnik, 1995).

New World primate predators were classified into three categories based on predator types (i.e. birds, mammals and snakes) reported in the scientific literature, which provided the basis for our predator image database. Bird predator genera were *Accipiter, Aquila, Busarellus, Buteo, Geranoeatus, Harpia, Leptodon, Leucopternis, Micrastur, Morphnus, Parabuteo,* and *Spizaetus*. Mammal predator genera were *Panthera, Leopardus, Puma, Herpailurus, Leopardus,* and *Eira*. Snake predators included individuals from the families Colubridae, Elapidade, Boidae, and Viperidae. Each genus or family were equally represented in each category totalling 100 images per group downloaded from Google Images©, totalling 400 images, 300 from predator categories plus 100 from the control group. Each of the three categories contained 100 full-body images of similar sizes against a naturalistic background. We also used aircraft images downloaded from the Caltech 101 database as a control group (Fei-Fei, Fergus, & Perona, 2007) (Figures 13 and 14).

A

B

C

D

Figure 12: Sample images from the full colour image dataset (A – Mammal; B – Bird; C – Reptile; D – Aircraft)

A

B

C

D

Figure 13: Sample images from the colour blind image dataset (A – Mammal; B – Bird; C – Reptile; D – Aircraft)

All images for the colour based models were transformed to L*a*b* colour space, which consists of a lightness channel, a red-green opponency axis and a blue-yellow opponency axis (Appendix II) (McLaren, 1976). This allowed us to remove luminosity from the analysis, thereby leaving only colour to classify the predator type and identify variations of similar colours. Each image was reduced to 16x16 pixel cells where the average colour was computed and used to produce the features to be determined (Appendix III).

We used the Computer Vision System Toolbox in Matlab R2016 to create a collection of image descriptors. Each descriptor consists of a subset composing features from the original image by the command “bagOfFeatures” (Csurka, Dance, Fan, Willamowski, & Bray, 2004). A total

of 1000 descriptors for each predator image was obtained using a custom extractor based on colour information. All descriptors were automated selected, which consisted in patches of the processed image varying in sizes of 32, 64, 96, and 128 pixels.

The same methodology was repeated for the same 400 image dataset adjusting the red-green colour information by using colour blind simulations using the Vischeck Plugin (Dougherty & Wade, 2006) in ImageJ software (Rasband, 2016). Thus, a dichromatic (colour blind) simulation image dataset was created and used to train the dichromatic colour vision classifier (Figure 15).

Using the same training full-colour image set we employed a shape based model, Speeded Up Robust Features (SURF), to classify the images (Bay et al., 2006). The same method as previously described was used and validated as per our colour based models.

The performance of the models was evaluated by a Confusion Matrix and ROC (Receiver Operating Characteristic) curve graph (Fawcett, 2006). The Confusion Matrix is used to compare the predicted classes to the true class (Sammut & Webb, 2011). The ROC illustrates a trade-off between sensitivity and specificity while closer the curve is to the top of the true positive axis, the more accurate is the model. If the area under the curve is close to one it represents a very accurate model, while an area of 0.5 represents an unsuccessful model (i.e. random assignment of classes).

5.2.2 Applying classifiers with a camouflage image dataset

We also built a 100 image dataset to experiment and validate the model containing 25 images of each category with camouflaged animals (i.e. matched background) or partially hidden downloaded from Google Images©. The control group Airplanes had images of

camouflaged aircrafts. We verified the accuracy of the three models created to access the performance of different phenotypes.

The Kappa with linear weighting was used to verify the agreement between the true and predicted class if attributing categories by chance. Next, each model was compared using Z-test evaluating the differences between each of the three trained models. Lastly, to compare the performance in identifying camouflaged targets, a Chi-squared test was performed comparing the two possible outcomes (i.e., correct and incorrect assignment).

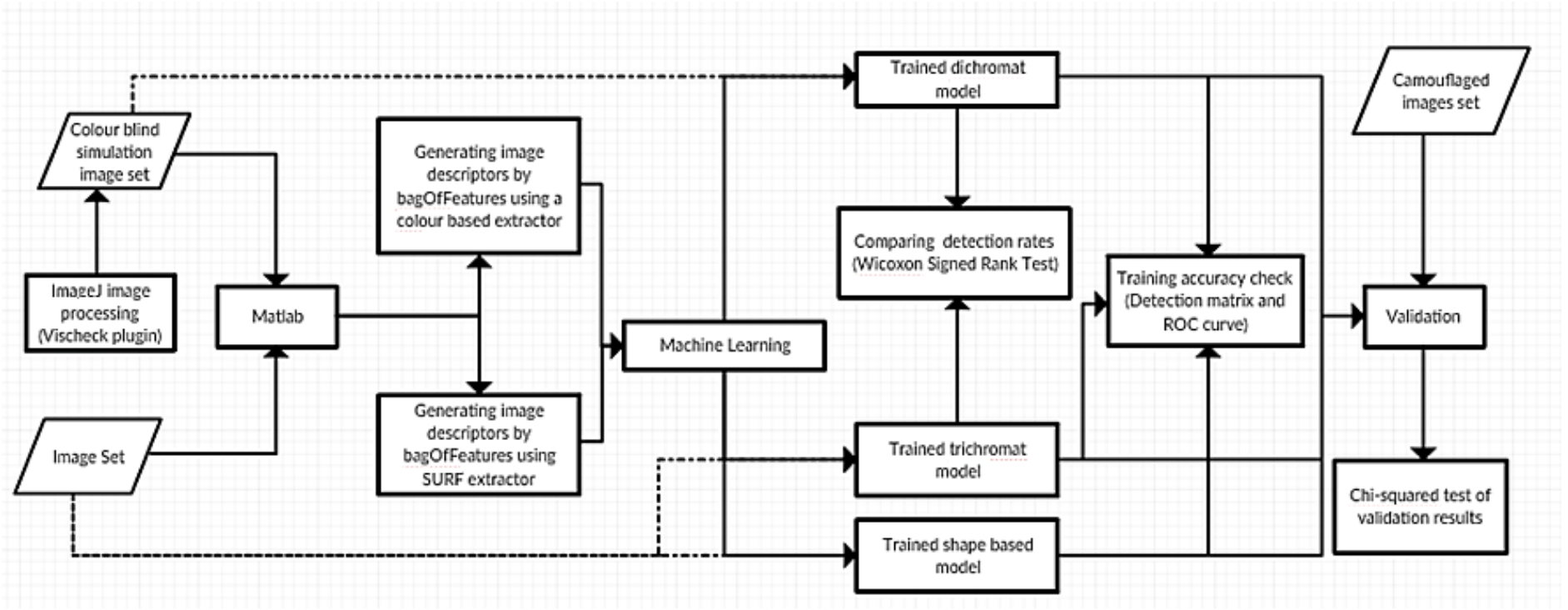


Figure 14: Workflow for model training, validation and detection of camouflaged targets

5.3 Results

5.3.1 Model training

The resultant trained image classifier from the trichromatic image predator dataset had an accuracy of 68.8% with the Ensemble Bagged Trees classifier method; this classifier consists of a bootstrap method using randomly generated training sets to attribute the correct category. The most distinctive category was the control group (airplane) with 97% of correct identifications from the dataset, followed by birds with 65%, mammals with 59%, and snakes 54%. The most distinguishable category was the control group with 3% error rate. The most confused categories were mammals and snakes, where 24% of mammals were identified as snakes and 28% of snakes were identified as mammals (Figure 16). This colour based model trained with the full-colour image dataset correctly assigned 68.75% of the observations where 25% represents the number of agreements expected by chance (K=0.674, SE= 0.028, CI=0.61-0.72).

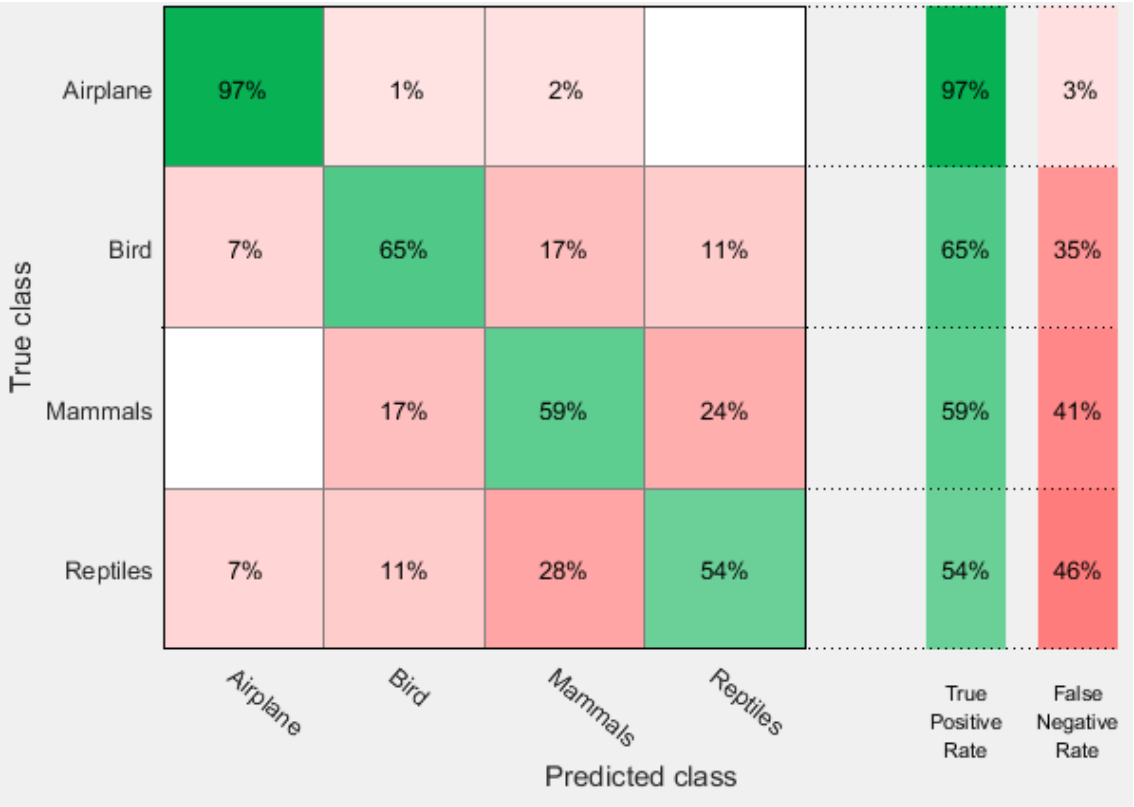
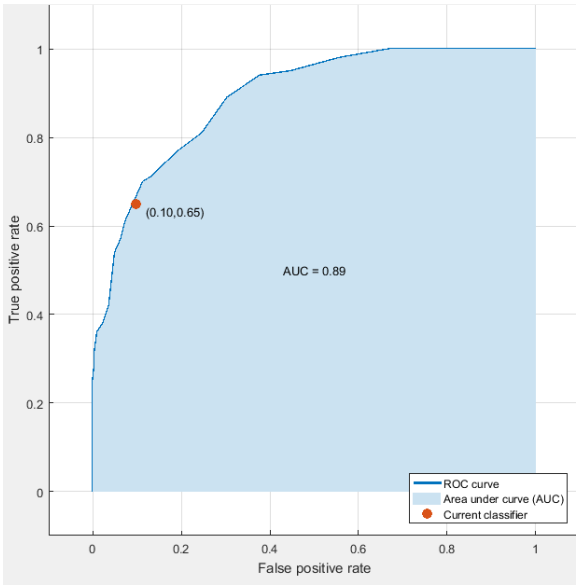


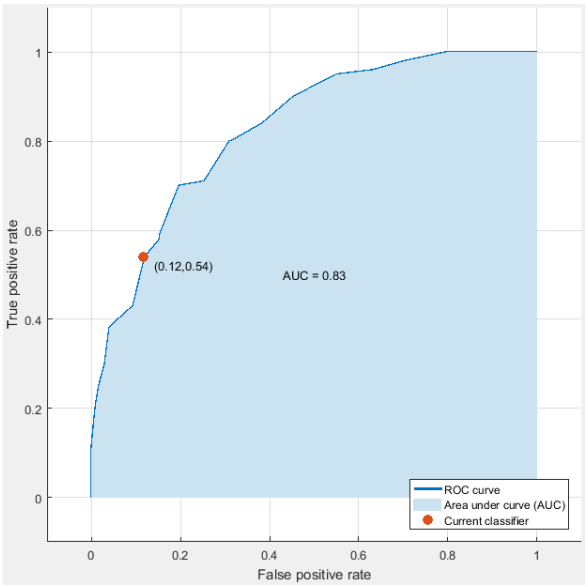
Figure 15: Confusion matrix for the trained colour based model with the full-colour image dataset.

By analysing the ROC diagram it is possible to verify the accuracy detection for individual classes (Figure 17). The current classifier had a good performance in identifying the control group with 0.99 accuracy (AUC=0.99). In the predator categories, the classifier had a 0.89 accuracy for birds, 0.84 of mammals and 0.83 of snakes. Thus, it was possible to use colour to correctly identify predators based only on colour information.

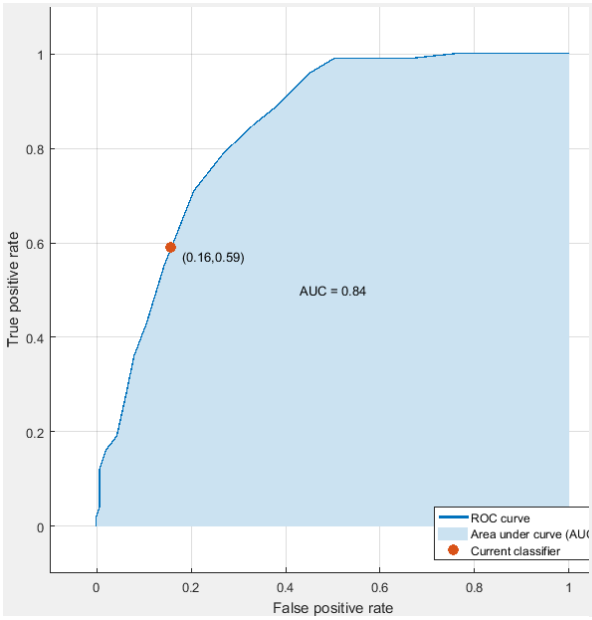
Bird



Snakes



Mammal



Airplane

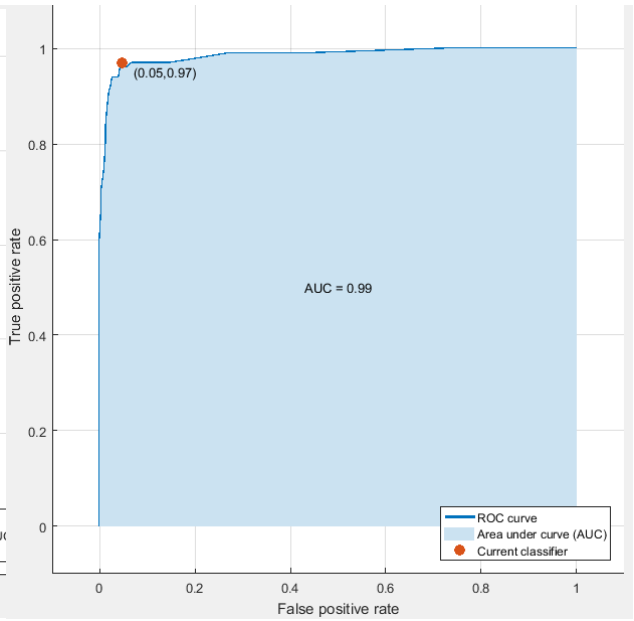


Figure 16: Receiver operating characteristic (ROC) curve for the trained colour based model with the full-colour image dataset

The dataset of colour blind simulations was used with the same colour based method to create the dichromatic colour vision classifier (Figure 18). We found a 61% overall accuracy with the Ensemble Bagged Trees classification method. The control group had a 94% true

positive discovery rate. The trained classifier was able to identify 60% of the bird category and 45% of mammals and snakes. Similarly to the full-colour dataset, a confusion zone occurred where 38% of mammals were identified as snakes and 35% of snakes were identified as mammals. This model assigned 61% of the categories correctly (K=0.60, SE=0.025, CI=0.5422-0.6578).

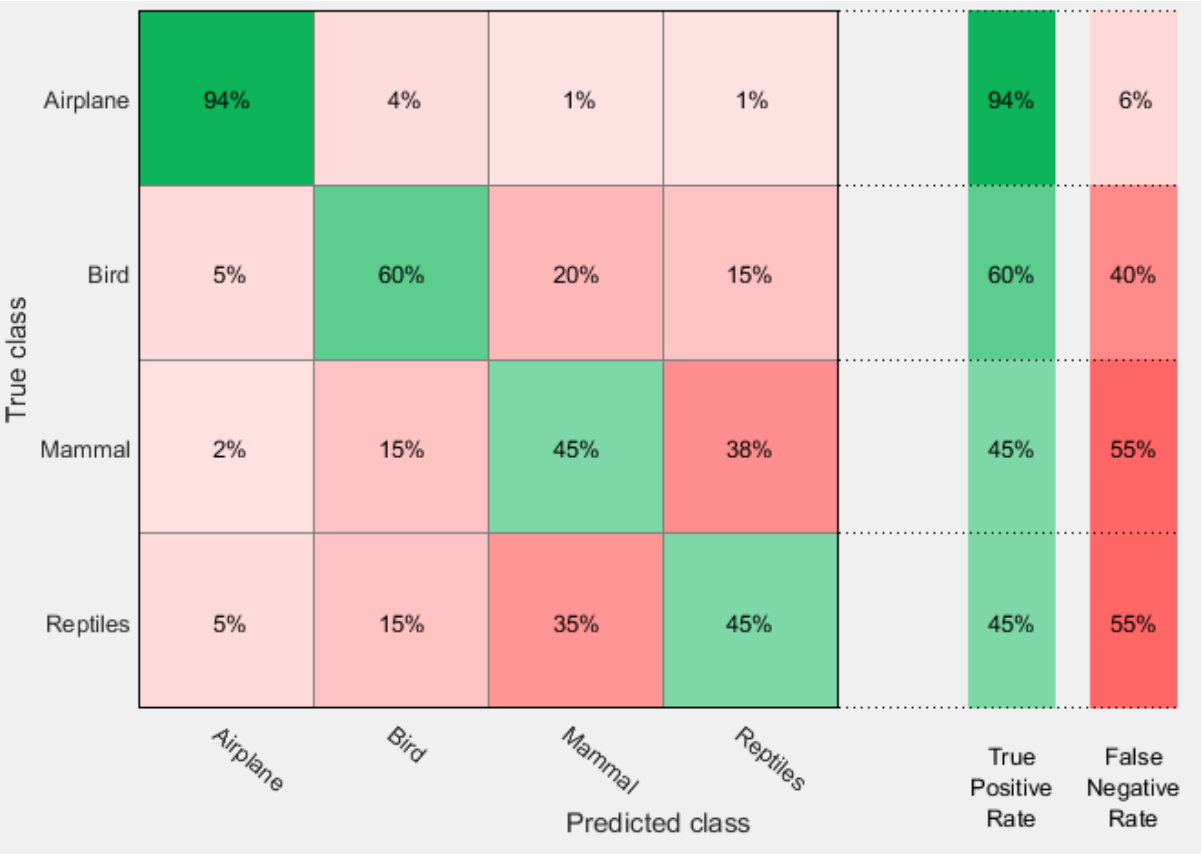
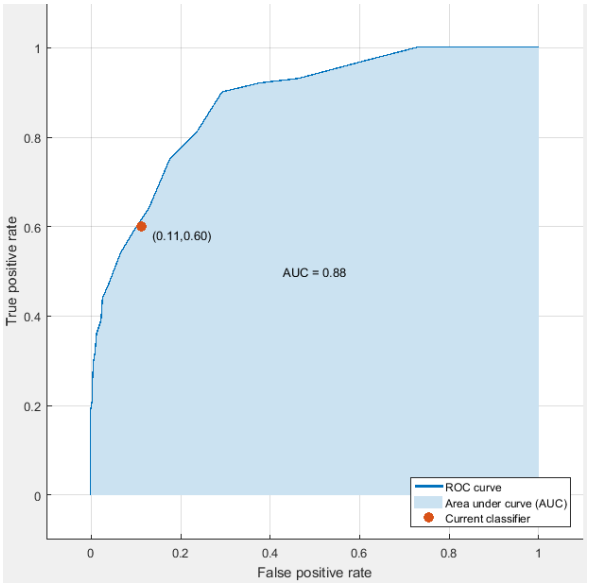


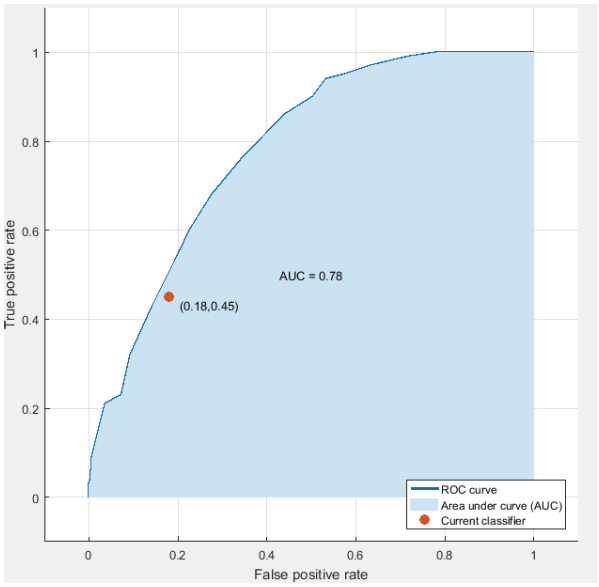
Figure 17: Confusion matrix for the trained colour based model with the colour blind simulation image dataset

The accuracy of the trained classifier for each category separately was lower when compared with the classifier trained using full trichromatic colour images. However, both had an accuracy of 0.99 in identifying the control group (airplane). The trained model with the colour blind image simulation dataset had a performance of 0.88 for the bird category, 0.78 of snakes and 0.77 of mammals (Figure 19).

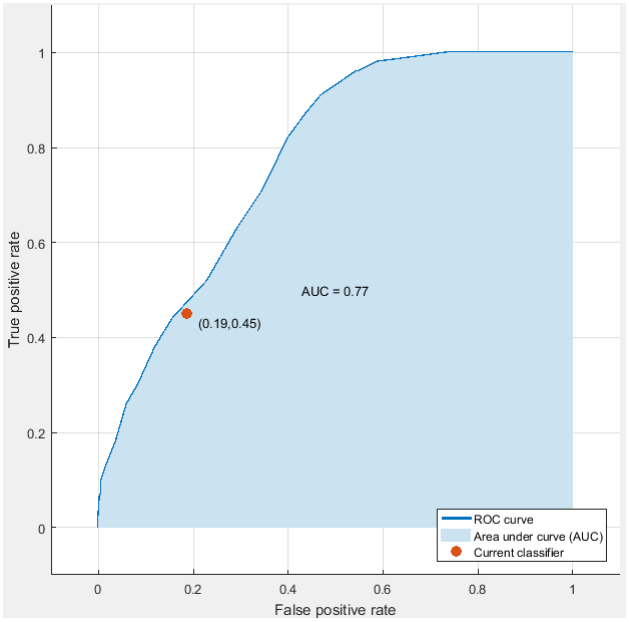
Bird



Snakes



Mammal



Airplaine

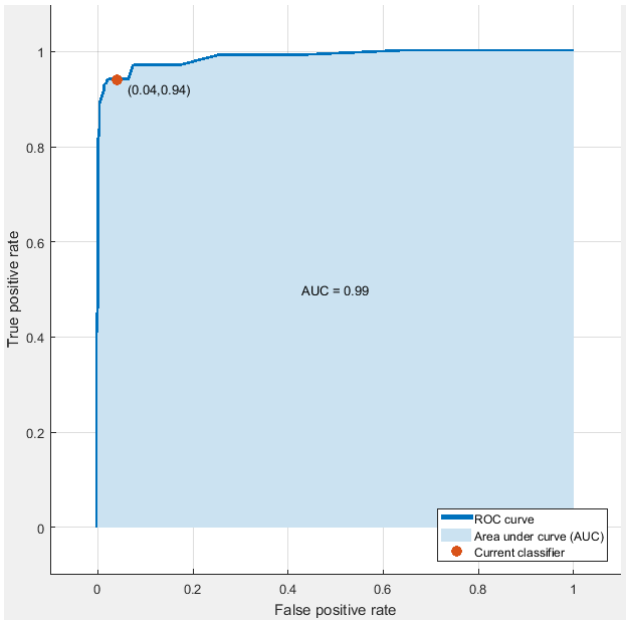


Figure 18: Receiver operating characteristic (ROC) curve for the trained colour based model with the colour blind image dataset

The best classification results were achieved using the shape based classification method. The best classifier was the Support Vector Machine Linear with an accuracy of 78%. As a result, 99% of the control group were correctly assigned, 68% of the bird class, 74% of the mammal

and reptile class (Figure 20). We found the best agreements between the true and predicted predator classes with the shape-based model with 78.75% of the images correctly assigned (K=0.8834, SE=0.0097, CI=0.8644-0.9024).

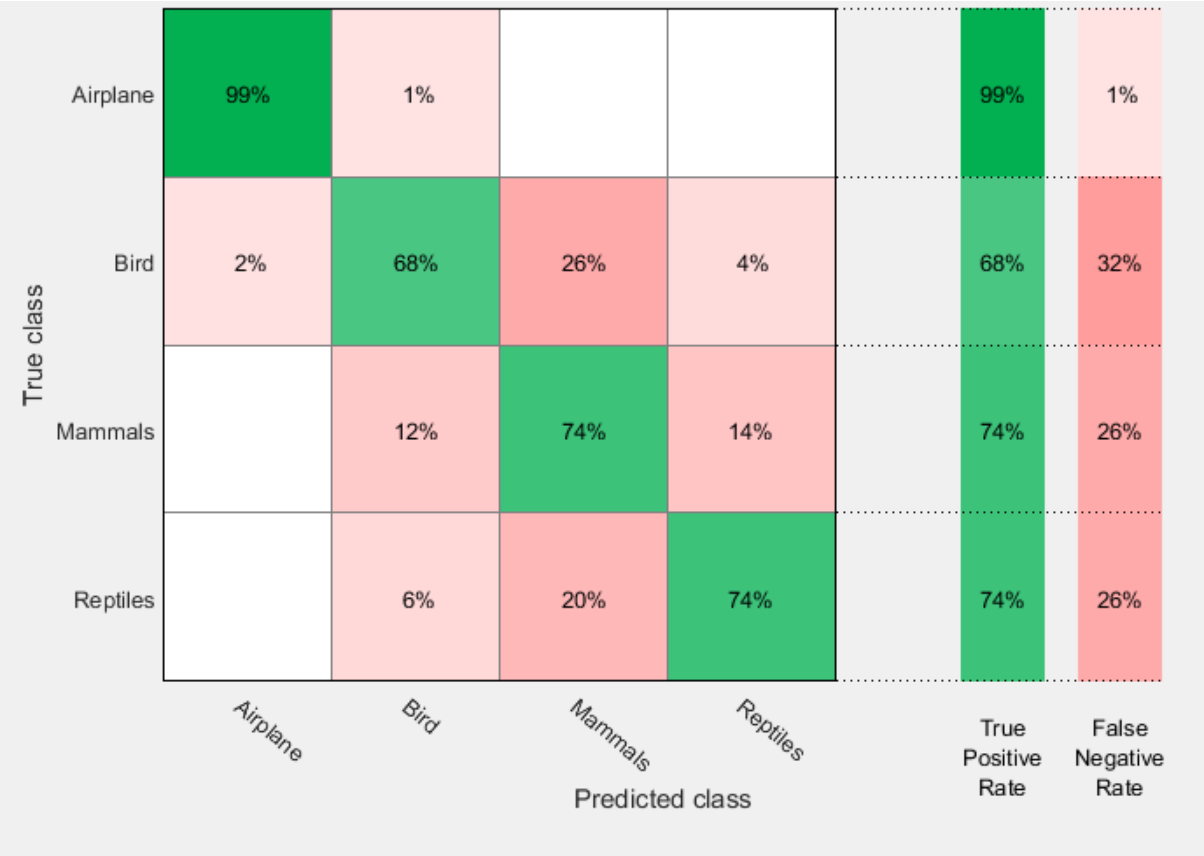
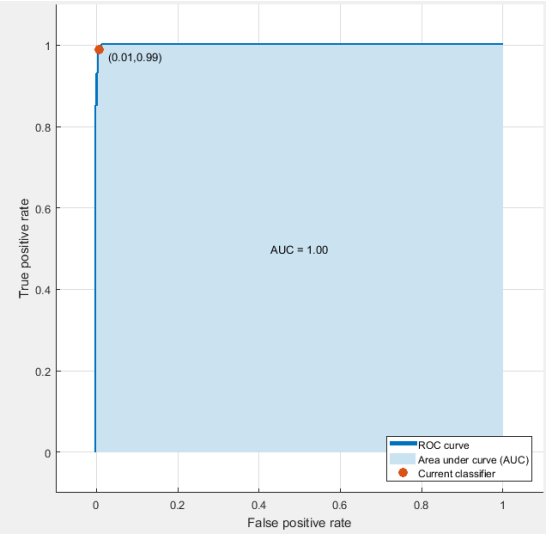


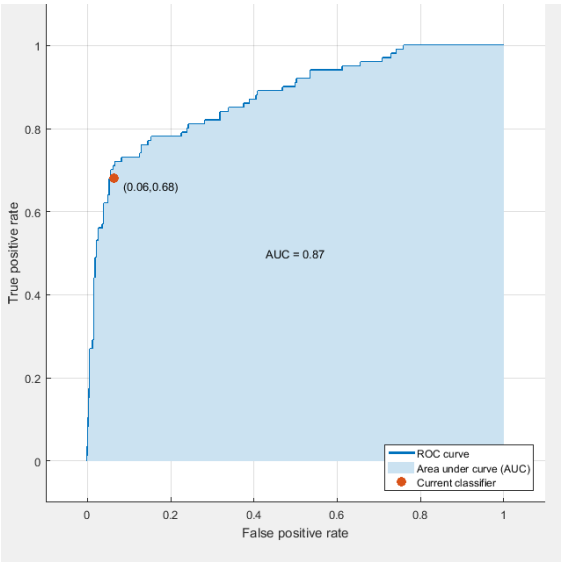
Figure 19: Confusion matrix for the trained shape based model trained with the full-colour image dataset

Similarly, the highest area under the curve values were found using the shape based classification method with the control group with an AUC of 1, snakes with 0.92, and birds and mammals with 0.87 (Figure 21).

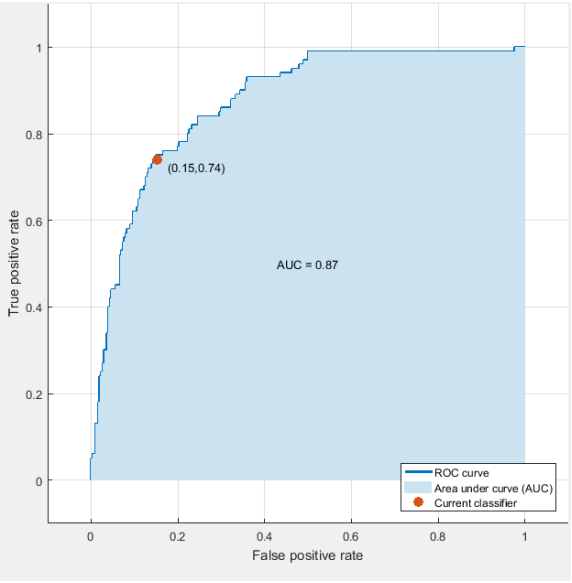
Airplanes



Bird



Mammal



Snakes

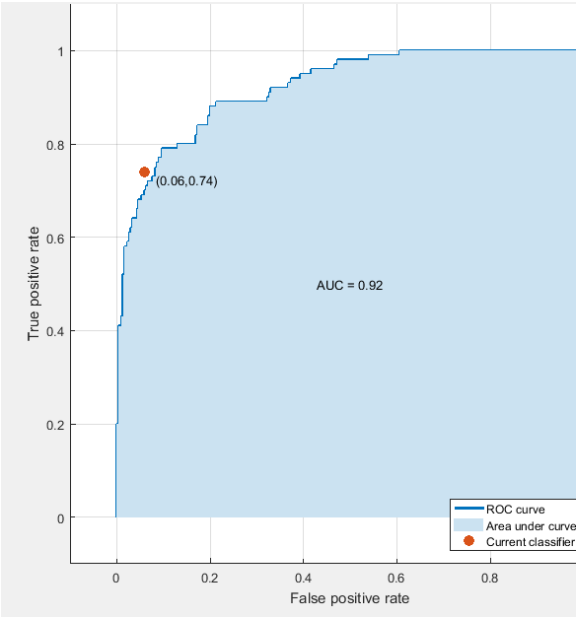


Figure 20: Receiver operating characteristic (ROC) curve for the trained shape based model with the colour blind image dataset

We found no significant difference between the full trichromatic colour and the colour blind simulations trained classifiers, the performance of the models was similar in identifying predators correctly (Z=1.866.181, P=0.8564). However, the shape based model differed significantly from the trained model with full-colour images (Z=-7.2095, P=0.00001) and with

the colour blind simulations image set (Z=-55.3507, P=0.00001). In summary, colour based models (i.e. Trichromat and Dichromat) have a similar performance and are able to identify predators based solely in colour; however, shape was a more relevant/important source of information when classifying predators.

5.3.2 Applying classifiers with a camouflage image dataset

By using the trained models with an image data set of camouflaged targets we experimented the efficiency of the models camouflaged targets. The trained colour based model with a full-colour image dataset, produced 43% of the images assigned to the correct category, which represents the detection of a trichromatic colour vision phenotype. The model trained with the colour blind image simulation, representing the dichromatic colour vision phenotype, had 24% of correct assignments. Comparing the results, we found a significant difference in the experimentation results between the colour based models (χ^2=8.102, DF=1, P=0.0044). Thus, the trichromatic model outperformed the dichromatic model in identifying camouflaged animals and the control group.

Applying the shape based model with an experimental set of camouflaged images, we found an accuracy of 39% of correct assignments in the predator category and control group. By comparing the validation of the shape based model and the colour based model we found that there was no significant difference between the shape and trichromatic colour classifiers in detecting camouflaged images (χ^2 = 0.331, DF=1, P=0.5652). However, the dichromatic colour classifier had a lower performance when compared with the shape-based classifier (χ^2 = 5.214, DF=1, P=0.0224).

5.4 Discussion

These models offer a generic model to investigate the importance of colour information in predator recognition and detection. Given the high accuracy with the control group this demonstrates predators as a broad category, which was recognised in our image data-base sample. In fact, the trained classifier showed that it is possible to classify primate predators reliably using only colour characteristics, in both dichromatic and trichromatic colour vision phenotypes. The highest accuracy was for the control group this certifies that the model is accurate in distinguishing the control from the Bird, Mammal, and Snake categories. A confusion zone was observed, mainly between mammals and snakes. This was observed for both trichromatic and dichromatic models. The shape based model also had a similar performance when compared with both dichromatic and trichromatic models.

The importance of shape and patterns in identifying a target has been one of the main justifications for the evolutionary persistence of dichromatic colour vision. For instance, the ability to break through camouflage is referred to as one adaptive advantage of defective colour vision (Melin et al., 2007; Morgan et al., 1992; Saito et al., 2005). Indeed, mammals preyed upon by camouflaged predators are generally dichromats and yet are still able to present an adequate anti-predator behavioural response (Bowmaker, 2008; Yokoyama & Radlwimmer, 1999). However, trichromats can outperform dichromats in detecting camouflaged animals (Pessoa et al., 2014). In addition, some small mammal species have developed resistance to snake venom to resist snake predation attempts thereby decreasing the importance of visual recognition (Isbell, 2006; Pérez & Sánchez, 1999). We found the dichromatic and the trichromatic colour vision model had similar training performance. Thus,

the loss of the red-green colour channel is not a definitive feature that increases predator categorization, but it was relevant in detecting camouflaged targets.

When using the trained model to detect predators that were partially hidden or camouflaged in our image dataset the performance dropped drastically in all models, as expected. However, the model trained with colour blind simulation images had a significantly lower performance. Therefore, no evidence of advantage in dichromatic colour vision phenotypes breaking through camouflage was found. We found a difference between the shape based model and the colour based model, which means that colour and patterns have different implications in predator detection. Therefore, despite the ability to recognise predators, it was not possible to separate camouflaged animals or controls.

Although not investigated here, dichromatic colour vision is more suitable to identify targets in low light levels (Verhulst & Maes, 1998). For instance, the nocturnal primate *Aotus* lost the short-wavelength sensitive opsin, thereby presenting a monochromatic colour vision (Curtis & Rasmussen, 2006; Donati & Borgognini-Tarli, 2006; Jacobs, Deegan, et al., 1993). This fact itself demonstrates the correlation between light levels and colour vision. Cathemerality itself does not reduce predation as primate predators might also be nocturnal; however, the reduction of predators, such as raptors, is an important selection pressure for nocturnal activity (Colquhoun, 2006). The low performance of the dichromatic colour vision model with the camouflaged predator images demonstrates that dichromatism might be used for other purposes. As a consequence, the importance of scotopic vision for New World primates might be more relevant than the detection of camouflaged targets in bright light for dichromatic colour vision phenotypes. Environmental light is an important component, which can interfere with colour perception. Polymorphic colour vision species make use of the different light levels in the forest shade to forage on green and non-green foods, while full trichromatic

colour vision species make no distinction when feeding on different food colours in varying light intensities (Yamashita et al., 2005).

When identifying camouflaged predators, the red-green colour was found to be relevant for the identification of predators with results similar to that achieved with the shape-based model. This means that colour and pattern information might work together to better identify camouflaged predators or prey in trichromatic colour vision primates allowing a startle response. This is in agreement with Isbell (2006) who found that the evolution of the complex vision system found in mammals is related to coevolution with predatory snakes, where a fast and subconscious response via pre-cortical processing involving contrast and colours is essential for survival.

Colour and contrast have complementary advantages in predator recognition allowing a rapid behavioural response in primates. Between the two main hypotheses for the adaptive value of dichromatic colour vision: in breaking through camouflage or scotopic vision, the last would be most relevant for New World primate species. Further studies should design experiments considering the interaction of colour and contrast (i.e. shape and patterns) to evaluate the response to camouflaged objects rather than comparing dichromatic and trichromatic colour vision phenotypes.

Chapter 6 -Behavioural Measurement of Colour Blindness in Zoo-Housed Primates: A Proof of Concept Study

Abstract

Zoos are an important repository of animals, which have a wide range of visual systems providing excellent opportunities to investigate many comparative questions in sensory ecology. However, behavioural testing must be done in an animal welfare friendly manner, which is practical for zoo staff. Here we present a proof of concept study to facilitate behavioural research on the sensory ecology of captive animals. A colour blind testing system consisting of a tablet computer and an automated feeder connected wirelessly was developed and presented to captive primate species to evaluate interactions with and without previous training. A colour stimuli, similar to Ishihara test, was produced and checked for colour blind discrimination through sensory analysis. Animals were able to use the system successfully and displayed signs of learning to discriminate the stimuli presented. From this study we have further learnt the steps necessary to produce a fully automated system for measuring the visual abilities of zoo-housed animal species. We conclude that zoos are a suitable environment for conducting such behavioural research without major alterations in the animals' management routine or enclosure. We identified no risk for animals in allowing interaction with the experimental setup without the presence of keepers. In the long-term the system developed here will allow us to address complex comparative questions about the functions of different visual systems in captive animals.

6.1 Introduction

Zoos are an important repository of animals housing tens of thousands of different animal species around the world and millions of individuals, providing environmental education, aiding conservation efforts and contributing to scientific research (Barongi, Fisken, Parker, & Gusset, 2015; Patrick, Matthews, Ayers, & Tunnicliffe, 2007; Tribe & Booth, 2003). Zoos have a great potential for behavioural research, but for pure research, such as sensory ecology, this resource is largely untapped. The most obvious explanation for this is that zoos focus on conservation research, but also zoos have many other restrictions on the type of research they will allow (Kleiman, 1992). For instance, a challenge faced by researchers in conducting studies in zoos is the restriction on handling animals or modifying the enclosures, which is often necessary to achieve the best experimental design (Hosey, 1997). Furthermore, zoo-based research should not impact negatively on animal welfare or consume great amounts of human resources. Given that these restrictions are red-lines that zoos will not cross then researchers need to develop alternative methods for conducting their research.

Computer interfaces are centred in human abilities, such as the visual system and physical input hardware (i.e. keyboard or mouse); however, some interfaces are cross-species, such as audio, video tracking, accelerometers, and haptic sensors (McGrath, 2009). This leads to the development of interfaces appropriate for animal use. Animal-computer interaction (ACI) is a growing field in the environmental enrichment and animal experimentation fields (Ritvo & Allison, 2017). It can be a source of stimuli in animal enclosures providing an enriched environment for better animal welfare (Wirman & Zamansky, 2016). Moreover, this approach can be used to experiment and test hypotheses, thereby allowing the study of animal behaviour in animal collections such as zoos (Mancini, 2011).

Tablet computers are an affordable technology to investigate the sensory abilities of different animals: their thin profile and touch interface allow an intuitive interaction when compared to traditional computers where mouse and keyboards are the main input hardware. Therefore, there is no need to translate physical movement into virtual movement (Ritvo & Allison, 2017). The number of sensors present in commercially available tablets increases the possibility of their application to address behavioural and sensory questions. For instance, accelerometers built in the device can be used to record positional behaviour (Graf et al., 2015); proximity sensors can trigger data loggers (Nathan et al., 2008); or the display and touch screen can be used for stimuli presentation (Takemoto et al., 2015; Takemoto, Izumi, Miwa, & Nakamura, 2011).

One challenge imposed in using touch screens for colour stimuli presentation is that colour replication accuracy cannot be guaranteed. For instance, sensory ecology vision research usually employs no digital compression in photos taken from cameras and colour checker cards or colour referenced stimuli (Melin, Kline, Hickey, & Fedigan, 2013; Pessoa, Cunha, Tomaz, & Pessoa, 2005). Therefore, any behavioural research should take in consideration variations in colour stimuli presented on different screens type and proceed with calibration methods.

Colour vision is varied among animals. Colour discrimination is related to the number of photoreceptors sensitive to different wavelengths of light (Shozo Yokoyama, 2000). Vertebrates, such as the nocturnal owl monkey (*Aotus sp.*) have one photoreceptor and are not able to distinguish colours (monochromats); most mammals have two photoreceptors are dichromats; the majority of Old World primates are trichromats and have three photoreceptors; fishes, reptiles, and birds are tetrachromats and have the best colour vision acuity among vertebrates (Bowmaker, 2008; Jacobs, 2009). The greatest number of

photoreceptors is found in the mantis shrimp (*Neogonodactylus oerstedii*), which has 12 photoreceptors (Thoen et al., 2014). The animal-computer interface display used to perform behavioural research should be appropriate to the colour vision system of the species in terms of the colour discrimination tasks involved.

New World primates have a polymorphic colour vision system (Hunt et al., 1998; Jacobs, 2007). Within the same species, males are obligatory colour blind with a colour vision similar to a red-green colour blind human, whereas females can be either dichromats or trichromats (De Valois & Jacobs, 1968; Dulai et al., 1999; Jacobs et al., 1996). Each phenotype has its advantages: dichromats are best suited for vision in low light levels and breaking through camouflage, whereas trichromats outperform dichromats in detecting ripe food sources or detecting predators in photopic light levels (Dominy & Lucas, 2001; Morgan et al., 1992; Osorio & Vorobyev, 1996; Pessoa et al., 2014; Verhulst & Maes, 1998). Despite the advantages of each phenotype, the importance of having both phenotypes in the same group is still to be investigated. Some hypotheses have been generated, such as heterozygotic advantage, niche divergence and mutual association benefit, despite this, not one of these hypotheses has been able to provide a definitive answer. Heterozygotic advantage could maintain both phenotypes in the population; however, a long-term study does not confirm this hypothesis as it was not confirmed by survival rates and reproduction of individuals in the group (Fedigan et al., 2014). Another potential explanation for the polymorphic colour vision in New World primates is niche divergence; again, Melin and collaborators (2008) were not able to confirm this. Further studies investigating social cooperative behaviour related to visual perception would help us to understand the role of polymorphic colour vision in New World primates.

Here we present a proof of concept study concerning the realisation of behavioural vision research in zoos. Thus, we investigated the use of commercial devices (i.e. off-of-the-shelf

tablet computer) and the development of customisable feeder to be used by keepers in zoo enclosures without causing disturbances to animal management or animal welfare.

6.2 Methods

6.2.1 Animals

A mixed group of two marmoset individuals (*Callithrix geoffrey*), one male and one female, and three titi monkeys (*Plecturocebus cupreus*) a pair and a young male, kept in the same enclosure in Twycross Zoo, United Kingdom were subjected to experimental sessions. The experimental sessions took place in the same enclosure where the animals were housed, the animals were free to move to the outdoors. There was no need for handling or capture the animals during the study (Figure 22).

Figure 21: 360º view from inside the marmoset and titi enclosure while performing the experiment

A single group of three variegated spider monkeys (*Ateles hybritus*), two females and one male, housed also in Twycross Zoo were also used in the experiment. The experimental setup was placed in an animal management area, the animals being free to move to their habitual enclosure or outdoors during the experimental session; that is, and voluntary participation in the tests (Figure 23).

Figure 22: 360º view from outside the spider monkey the enclosure while performing the experiment

Animals were not deprived of food, and routine feeding and food enrichments maintained during the study. Experimental sessions were performed according to the keeper's routine husbandry sessions from December 2016 to April 2017. Each session lasted 10 minutes and would be terminated in case of any undesired circumstance (e.g. aggression) towards the apparatus or group members.

6.2.2 Stimuli

An image composed of circles in varying sizes and colours, similar to the Ishihara colour blind test (pseudoisochromatic plate) was produced using a Java code in Processing 2.2.1. Each circle had a maximum diameter of 22 pixels and minimum diameter of 8. A white background was set and the code produced a random pattern where a static figure in PNG file format was placed randomly on a canvas of 1024x576 pixels. The PNG file had no background and the outline and filling of the image were replaced by red-coloured circles. A rounded shape was used to produce a red target (Figure 24).

An Android app was created using MIT app inventor where the images created were shown on a tablet screen (Amazon Fire). If the target was touched, a clicker sound occured and the target's position changed. If the wrong area was touched, a horn sound was played and the target did not change its location. The tablet had a wireless connection to a feeder, which provided a food reward if the right area in the tablet's screen was touched. A red-green colour blind human volunteer checked if the target was visible or not. Also, a spectrophotometer, Ocean Optics USB 2000+ VIS-NIR, attached to a light source (LS-1 Tungsten Halogen light, Ocean Optics) was used to collect the relative irradiance from the tablet screen and the colours of the target and background were compared calculating JNDs (Just Noticeable Differences) modelling trichromatic and dichromatic phenotypes. Sensory analysis was performed using R and the package Pavo (Maia, Eliason, Bitton, Doucet, & Shawkey, 2013; R Core Team, 2016).

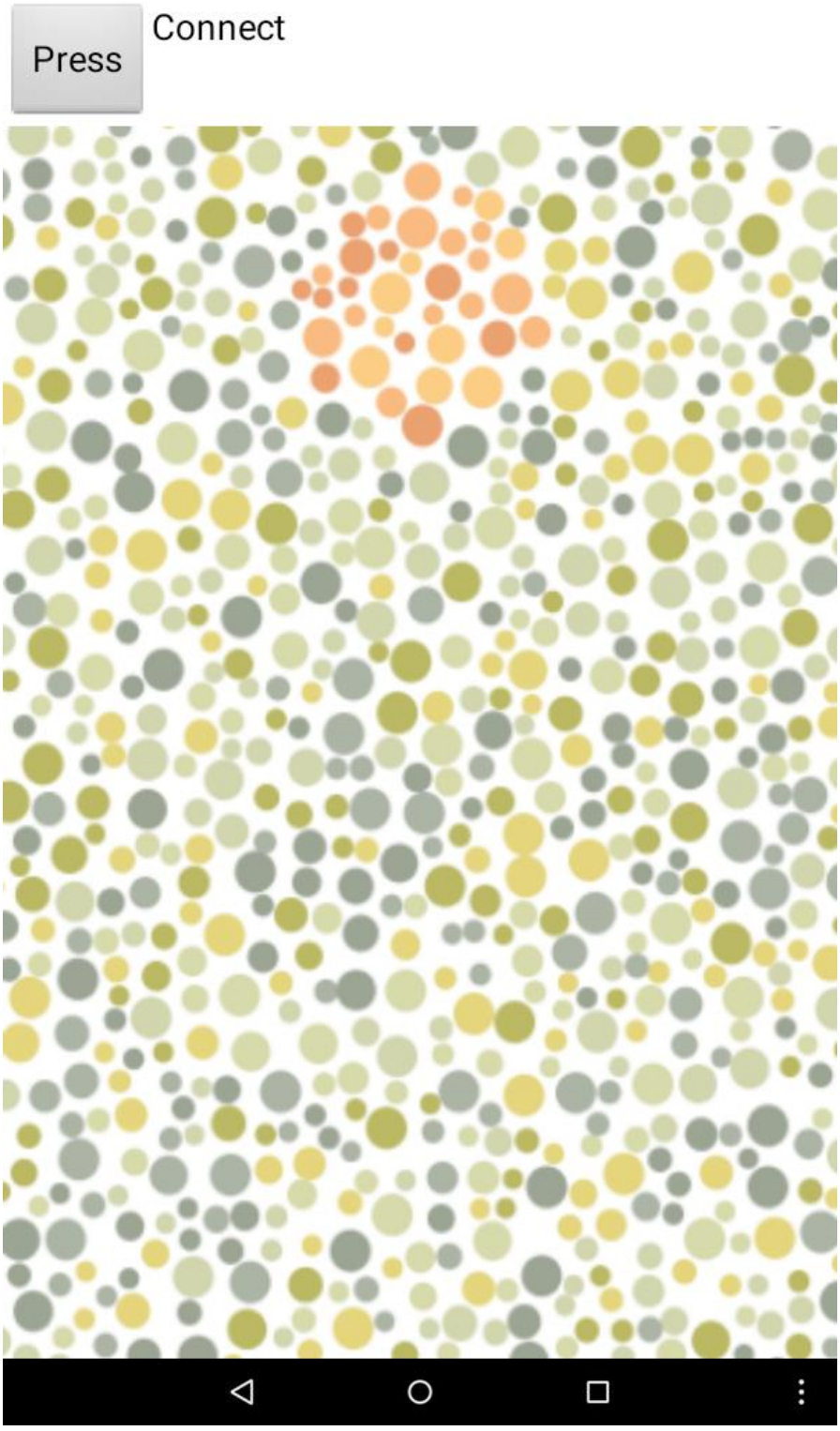


Figure 23: Screen capture from a colour stimuli created to investigate the interaction of captive primates with the apparatus developed to behaviourally investigate colour vision.

6.2.3 Apparatus

The feeder consisted of a DC motor attached to a plastic spiral that turns, pushing the reward (raisins) from a plastic container. An Arduino UNO microcontroller board (ATmega328P) was connected the DC motor using an L298N dual H-bridge DC motor driver module. An Hm-10 Bluetooth module was also connected to the Arduino allowing the wireless connection to the tablet. A Kindle Fire tablet with a 7-inch screen with a resolution of 1024 x 600 pixels and a rugged case was used (Table 12; Figure 25 and 26) (Appendix IV and V). A Ricoh theta 360 degrees camera in a protective case was used to film the experimental sessions.

Table 12: Components used to build a visual stimuli presentation device to behaviourally measure colour vision

Component	Cost
Kindle Fire tablet computer, 7-inch 1024x 600 screen, 313 grams	£35.00
Arduino Uno microcontroller board ATmega328P	£17.30
DC motor powered dispenser	£5.00
Bluetooth module Hm-10	£6.00
L298N dual H-bridge DC motor module	£5.00
Rugged tablet case with screen protector	£15.00

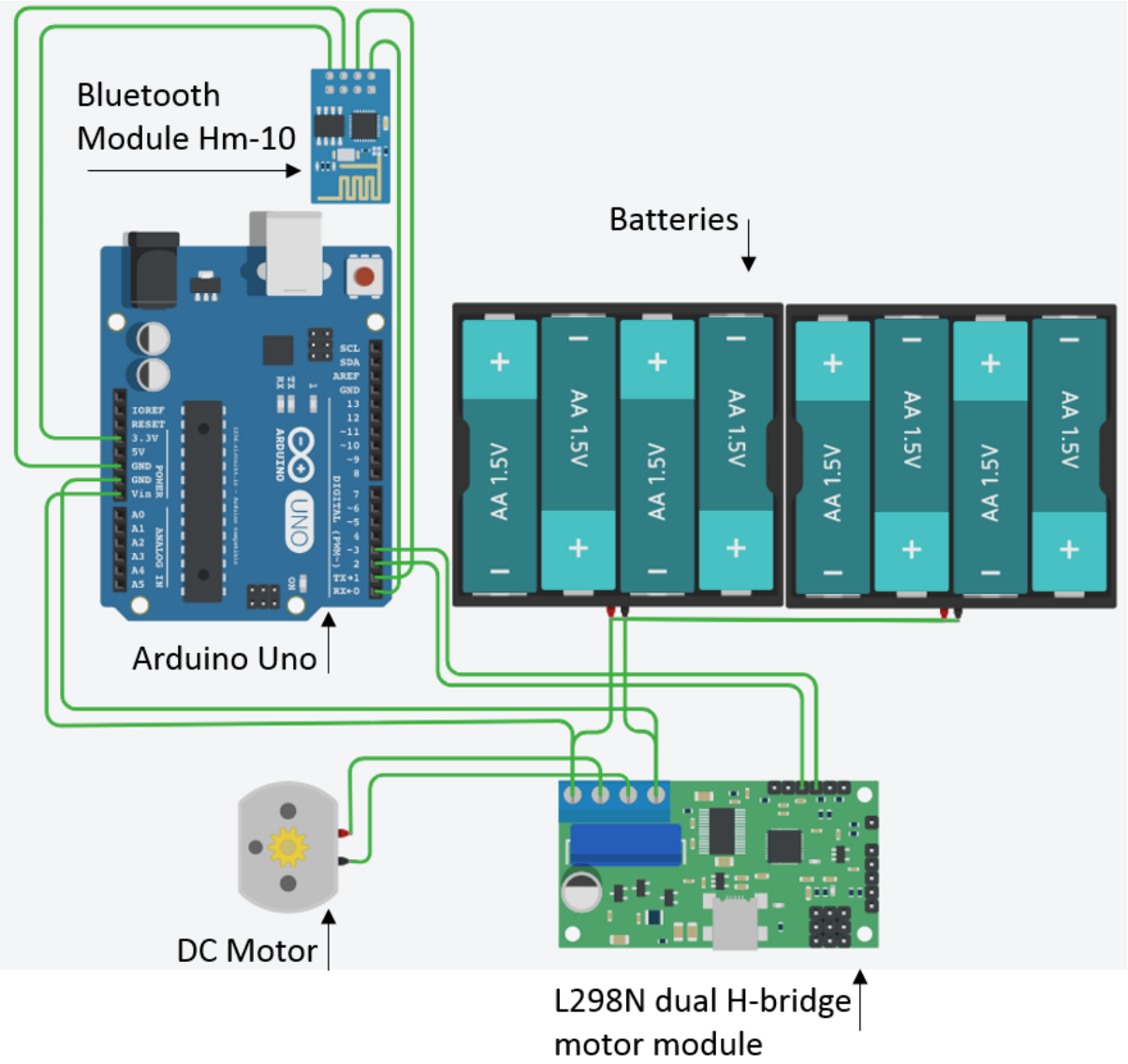


Figure 24: Schematic diagram of the automated wireless feeder used in behavioural colour vision research

Figure 25: Experimental apparatus during the performance of behavioural tests

6.2.4 Procedure

The feeder, tablet and the camera were placed inside the marmoset/titis enclosure during 10 trials. At the start of the session, a raising was placed on top of the correct stimuli to help the association between the red target and the reward. No training was performed with marmosets and titis.

In the spider monkey enclosure, the camera and the feeder were positioned outside the enclosure and the keeper held the tablet allowing the animals to touch the screen through the mesh. The training consisted in placing the raisins on top of the target during the first two minutes of presentation, leaving the animals free to interact with the tablet after the presentation. Behavioural data were collected *ad libitum*: reporting behaviours expressed during interactions with the device. The time and the result of the interaction were recorded. A total of 11 trials were performed.

The data were checked for normality and non-parametric tests were used as we found the data did not fulfil parametric requirements. A logistic regression was used to evaluate the association between the tablet and feeder, the number of sequential interactions between the tablet and feeder was related to the cumulative time of the experimental session in the marmoset/titi enclosure. The frequency of training (only with the spider monkey group) and the number of correct uses was checked for correlation to the accumulative time using a Spearman rank correlation test. All statistics were performed in R (R Core Team, 2016).

6.3 Results

6.3.1 Stimuli

The colours suitable for a colour blind test are shown in Table 13 and Figure 12. By averaging the target colours and background colour from the tablet screen using relative irradiance values, we found a colour distance of 2.8040 JND (Just Noticeable Difference) for a trichromatic phenotype, and 0.2849 JND for a dichromat viewer (Figure 27). Therefore, target and background were not distinguishable in colour for dichromat phenotypes.

Table 13: Colour references of the pseudoisochromatic stimuli found to be undistinguishable by dichromatic colour vision phenotype (i.e. red-green colour blind individual)

Colour category	HEX-Colour code	CIE-Lab L	CIE-Lab A	CIE-Lab B
Green	9CA594	66.6087	-6.3958	7.7143
Green	ACB4A5	72.3152	-5.5814	6.6789
Green	BBB946	73.3536	-13.7646	56.6906
Green	D1D6AF	84.3514	-8.2588	18.7266
Green	D7DAAA	85.7519	-8.8320	23.3500
Green	E5D57D	84.8571	-6.2664	45.3062
Red	EBA170	72.4431	22.2198	36.4731
Red	F9BB82	80.3436	15.6987	37.4752
Red	FCCD84	85.0087	7.6967	42.4568

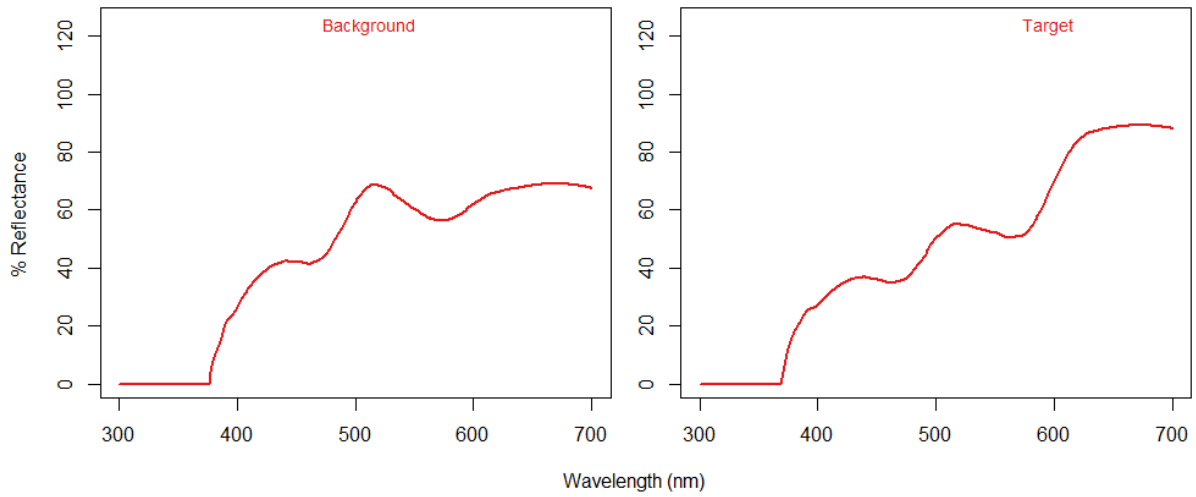


Figure 26: Relative irradiance of the pseudoisochromatic image taken from a commercial tablet (Kindle Fire).

6.3.2 Marmosets and Titis

A total of 82.52 minutes of stimuli presentation were obtained from ten experimental sessions. The device screen was sensitive to the touch of marmosets and titis (activated also by accidental touches, such as inspections and stepping on it). Interactions with the device had a mean time of 16.03 seconds (sd±16.89). The longest interaction took 101 seconds and the shortest lasted 2 seconds. Proportionally, 24.92% of the total presentation time was used in interactions with the device. Most of the interactions in the mixed species enclosure were performed by the marmosets (92.21%), who expelled the titis from the testing platform. We observed an association between the tablet and the feeder, with the animals inspecting the tablet and the feeder sequentially and looking to the display. From the experimental sessions, we did not find marmosets and titis using the device to receive the rewards. However, the individuals were associating the tablet with the feeder significantly according to a logistic regression ($\beta \pm SE = 0.001 \pm 0.0003$; $Z=4.131$; $P<0.001$). Unfortunately, it was not possible to assess the colour vision genotypes of individuals in the marmoset/titis enclosure.

6.3.3 Spider Monkeys

A total of 11 experimental sections were performed with a total interaction time of 66.68 minutes. All time was used by the experimental animals interacting with the device due to a different experimental setup from titis and marmosets. We assessed that spider monkeys were able to learn to use the device because they were able to receive rewards from the feeder five times in a row (i.e., no incorrect interactions). Agonistic behaviours were observed in two experimental sessions where the male, not being able to select the right colour target, repelled the female from receiving the reward. No collaborative behaviour was observed between males and females. The number of times that the keeper had to demonstrate and habituate the animal to use the device (i.e., training) was negatively correlated with the cumulative time of the experiment (r_s=-0.6245; N=11; P=0.0399). However, we found no correlation between the cumulative time and correct uses of the device (r_s=-0.2535, N=11; P=0.4520). Overall the experimental animals were participative and appeared motivated to use the experimental apparatus in the behavioural tests.

6.4 Discussion

The zoo found the device simple to use, it was easy to build and met the requirements for behavioural vision research in zoos. The touch screen of the commercial tablet with a rugged case and screen protector was reactive to the primates' touch. Even small primates, such as marmosets were able to interact and receive a reward. Therefore, ithe device is a suitable alternative to expensive scientific equipment used in some behavioural research (Takemoto et al., 2011). Efforts to bridge the gap between captive and wild animals research will provide more research opportunities and support an interdisciplinary approach (Shettleworth, 2001). For instance, research on colour vision requires the knowledge of physical properties of light, physiological aspects of colour vision, and implications of different phenotypes on the sensory ecology (Endler, 1993; Isbell, 2006; Stevens, 2013). Methodologies that allow us to access the sensory abilities of animal subjects are crucial to increasing knowledge in the field.

Two major hypothesis on the importance of polymorphic colour vision for New World primates are niche divergence and mutual benefit association (Hiwatashi et al., 2010; Melin et al., 2008). To understand if phenotypes have different niches or if animals can benefit from a mixed phenotype group it is necessary to expand behavioural research in sensory ecology. For instance, the niche divergence could be verified by changing the stimuli type from colour conspicuous to cryptic and observe the behaviour emitted by the different phenotypes. Alternatively, variation in light intensity could be used to investigate the role of photopic and scotopic vision in a temporal scale. The mutual benefit association hypothesis could be verified by investigating collaboration among individuals in response to a given colour vision task. The verification that primates can recognize abilities from other individuals in the group

(i.e., theory of mind) would suggest that cooperative behaviour is driving polymorphic colour vision in New World primate species. Testing these hypotheses are challenging due to difficulties in measuring behaviour related to the colour vision polymorphism and difficulties in studying of wild primates (Melin et al., 2012). Both problems could be reduced by studying captive primates with substantial collection of behavioural data.

Marmosets used in this study also inhabit forest fragments in cities, are frequently in contact with city dwellers and are often hand fed (Duarte, Goulart, & Young, 2012; Goulart, Teixeira, & Young, 2010; Teixeira et al., 2015); yet, they preserve many aspects of their natural behaviour (Duarte et al., 2012). The small sample sizes that often limit the value of research in zoos could be reduced by investigating urban animals. The portability of the setup used in this proof of concept study could easily be extrapolated to research in urban environments, which are more flexible regarding the access to animals and possibilities of modifying the environment. Marmosets in our study were able to associate the feeder and the tablet without assistance (training) from keepers. Thus, further studies with urban marmosets should be investigated.

One of our main goals with this study was to verify the use of zoo enclosures as an experimental area, having the keepers performing the experiment. Keepers have close contact with the animals and their interaction is relevant in designing scientific experiments (Carlstead, 2009). It was possible to shape the behaviour of spider monkeys with a few presentations of the experimental equipment. Nevertheless, preparation to perform the research and interest from the zoo staff are critical for success. Fortunately, Twycross Zoo is an institution interested in supporting behavioural research. Further presentations could be performed without the keeper's presence as the experimental setup allows its use without any human assistance. For monitoring purposes, the 360-degree camera we used allows

remote observation from any angle providing a surveillance method in real time (this camera can stream live video to a computer for recording).

As the opportunity to manipulate zoo enclosures is limited, certain precautions must be taken to ensure the successful realisation of experiments. We found that mixed species enclosures can lead to certain species not being able to participate in the experiments. This should be considered in future studies. A critical aspect of video recording is illumination. Indoor areas should be assessed regarding light sources, since the identification of the individual performing the behaviour is important in behavioural studies. Keepers often have a profound knowledge of each animal in their care, being able to recognise them without marking them. However, individual animal identification might be not possible if a 'control' researcher is used to analyse the recordings. For instance, if a double-blind experimental design is used, where the executive and analysis of experiments are performed by two different researchers independently.

Touch sensitive tablet computer screens can have different colour reproduction from the colour selected on the computer used for programming the tablet. However, we controlled for this by selecting colours that were not visible to dichromatic (human) colour vision phenotype. This problem is of major concern when developing colour based tasks in which the colour stimuli may be altered on different screens. Despite this, it was possible to use a commercial tablet to generate colour targets and backgrounds that are not detectable by colour blind viewers.

Unfortunately, due to time limitations, it was not possible to proceed with detailed behavioural studies of vision, but the concept was proved that a computer tablet based system can be used to behaviourally assess visual perception in zoo-housed primates.

Chapter 7 -General Discussion

Discussions about benefits of trichromatic over dichromatic colour vision help to understand why primates are the predominant order among mammals with more refined colour discrimination. This situation introduces the debate as to why polymorphic colour vision exists in New World primates. Likewise, trichromatic colour vision has been also reported in marsupial species (Arrese et al., 2006, 2002). The widely considered food detection hypothesis for the evolution of colour vision in primates does not match the occurrence of trichromacy in marsupials because closely related marsupial species with similar habitats and ecologies have either dichromatic and trichromatic colour vision (Ebeling, Natoli, & Hemmi, 2010).

The highly variable diet of primates and the constancy of polymorphic colour vision in the New World shows that food related hypothesis might not be the ultimate cause for the colour vision adaptations observed. In plathrryne species, it is possible to find frugivorous, folivores, seed eating specialists, and gummivores. Opsin frequencies in wild primate groups would shed light into the reasons concerning the most suitable phenotypes for each food type. As shown in Chapter 3, allelic diversity in opsins is high and with further studies might show the presence of all eight possible variations according to the “three-site rule” in other New World primate species. In fact, the total of all possible opsin allelic variations found across platyrrhines and prosimians was achieved by ten years of genetic based research and we predict that it will increase at the genus level (Figure 28). Thus, consequences of life style and diet could be responsible for changing allelic frequencies, allowing certain combinations of photopigments to be more common than others. Variation in diet might be responsible for

changing frequencies of opsins in polymorphic colour vision species, but might be not the ultimate cause for the polymorphism.

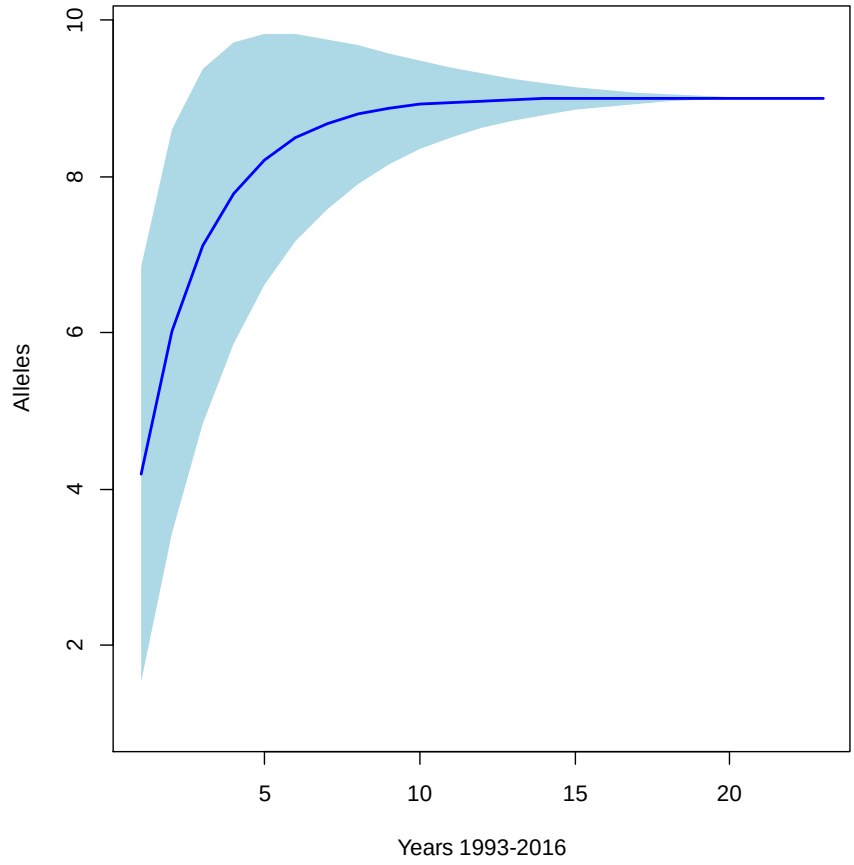


Figure 27: Accumulation curve of knowledge concerning medium-long wavelength sensitive opsins in New World primates and prosimians across time.

Legend: Dark blue solid line – Accumulation curve; Light blue shade – Confidence Intervals; Accumulation curve produced by recording the number of allelic variations reported on the scientific literature; Total number of records – 75; Results from Old World primates were not computed. Search engine – Web of Knowledge, Keywords – Primate* AND opsin AND polymorph*; Analysis performed with R using the package Vegan.

One distinctive feature of primate species is their social organization. Primates can establish long-term social bonds being distinctive patterns from other vertebrate species, because expressed social interactions have a significant effect in future relationships (Smuts, 1987). Social specialization often leads to sexual dimorphism. For instance, females that can breed with multiple male often develop perianal swellings or polygynous species are more sexually

dimorphic (Jungers, 2013; Nunn, 1999). By having males and females with different body sizes and features, frequently leads to niche diversification (Clutton-Brock, Harvey, & Rudder, 1977). New World primates lack sexual dimorphism when compared to Old World primates. Plathyrrines have a slight variation in body size between sexes and just a few species vary in pelage colouration, whereas catarrhines have mostly a pronounced variation in body size and other dimorphisms (Plavcan, 2001). As observed in Chapter 2, *Callicebus nigrifrons* shows a behavioural divergence in territory maintenance, foraging time, and parental care indication a niche divergence in this species. However, no sexual dimorphism is observed (Hershkovitz, 1990). What is known about sexual differences in this species is that it possesses a complex polymorphic colour vision system with a high allelic diversity.

Group living is related to predation risk (Alexander, 1974; Isbell, 2005). In Chapter 4, we demonstrate that predator risk and predator type affect the distribution of polymorphic colour vision primate species. While the routine trichromatic colour vision in *Alouatta* demonstrate no geographic distribution pressure from predator risk, polymorphic colour vision species avoided predator rich areas. *Alouatta* is the most widespread primate genus in the Neotropical region, which might be a consequence of the success of howler monkeys in coping with predation through their visual system (Crockett, 1998). Group size and body size are two well defined trends in primates; however, made little difference on a continental scale and including when phylogenetic signal was corrected for. Yet, polymorphic colour vision was the most relevant variable in explaining primate distribution in a predation risk gradient. This conclusion is corroborated by Chapter 5, where we found that the signals used for visual identification of predators were better achieved by trichromatic colour vision phenotypes than dichromats. Therefore, primate species with a mixed group of dichromatic and trichromatic individuals, independently from group size and body size, is a relevant

feature in determining species distribution. This suggests that different phenotypes within a group may cooperate in predator detection.

Evaluating cooperation in primates with different visual abilities is an important step to reveal the adaptive function of polymorphic colour vision. Given that dichromats and trichromats have different advantages in performing visual tasks, future research on cooperative behaviour will show how primates differ from other animals. For instance, mammals are mostly dichromats and are well suited for avoid predation, capturing animal prey, and finding food resources. Despite this, the polymorphic vision system has been maintained in New World primate species. In Chapter 6, a proof of concept to investigate the role of cooperation is presented. We believe that behavioural research on cooperation will define the advantage and mechanisms that maintain polymorphic colour vision in New World primates.

7.1.1 Limitations

In Chapter 2, we presented evidence of niche divergence between sexes as evidenced by behavioural differences related to light variations in the forest. Unfortunately, the assessment of the actual phenotype of each individuals was not possible due to failure to extract DNA from faecal samples. This limited our interpretation of the behavioural data. However, we were still able to detect differences in behaviour from males and females allowing conclusions about colour vision to be made. We believe that measuring sexual differences in behaviour is a suitable alternative for understanding the implication of colour vision polymorphisms, because molecular approaches are not always possible. We also did not measure light variations in the study site, thus our conclusions on light variation were deduced from time of the day and tree height use. In fact, to obtain ideal information on variation of light in the

foraging sites, it is necessary to measure light levels in the location of the feeding site and at the same time of feeding. It would not be possible to measure light levels in such locations without interfering in the behaviour of the study animals. Forest shade in different canopy levels is different in the Amazon and Atlantic rain forest due to variation in geographic relief. For instance, the steep hills found in the Atlantic rain forest would cause a variation in the canopy levels due to the different heights of the neighbouring trees (Endler, 1993; Koop & Sterck, 1994). However, we statically analysed the height of primates in trees in metres and the classification of the canopy level in the site and did not find any significant difference. Furthermore the study site did not show any physical characteristics to be consider at a local scale. Variations in light levels in forests in relation to tree height use and time of day are well described in the literature, which leads us to conclude that this variables are closely related, thereby justifying our analysis (Endler, 1993; Koop & Sterck, 1994; Théry, 2001).

In Chapter 3, the high allelic variation in Medium/Long wavelength opsin gene described has impacts on the knowledge of colour vision in Pitheciidae as it supports future research in the least studied primate family of New World primates. However, we were not able to investigate opsin frequencies in wild populations. This would help to further understand the implication of such diversity. For instance, the allelic variants found might not be common and the small sample size requires a confirmation from future research. Single nucleotide polymorphisms in sites in 180 in the Exon 3, 277 and 285 in Exon 5 are responsible for dramatic changes in predicted peak of spectral sensitivity (Kawamura, 2016). However, other sites in the gene with a smaller shifts in the sensitivity peak could add up causing a significant change in colour perception (Shyue et al., 1998). Despite this, studies with sites 180, 270, and 285 are widely accepted in reporting opsin alleles. The results found here

provide supporting information for whole gene genetic studies, microspectrophotometry of reconstituted opsin photopigments, and electroretinograms from living animals.

In Chapter 4, we discuss predation risk implications for the polymorphic colour vision system in New World primates on a landscape scale, but it was not possible to measure actual predation rates. This is a common problem with predation studies because predation events are rarely observed (Hart, 2007). The alternative we found was to measure predation risk by counting the number of predator species in a given geographic region as reported by species' distribution and assumed a higher predation risk where there was greater predator richness. Animal distributions are often considered as overestimated since the overall geographic localities where the species is found is reported (Gaston & Fuller, 2009). Also, the lack of data on snake distribution challenged the definition of the snake predators selected for the biogeographical model. In fact, all species from four venomous snake families were selected, whether predators or not. Given the strong response that primates show towards snakes and the phylogenetic approach used, we believe that the method and analysis employed were suitable to conclude the interactions between both predator and prey (Isbell, 2006). Predator vulnerability was not measured but should ideally be assessed. For instance, large-bodied primates being less vulnerable to mammal predators, whereas small-bodied primates are more vulnerable to raptors. However, limitations of raptors being able to carry primate prey, and the correlation on predation rate leads us to assume that variation in vulnerability to predator types is true (Ferrari, 2008; Isbell, 2005). We found no relationship between primate group size and predator type, but we do not rule out this possible relationship as predation events were not recorded. However, the most significant variable explaining primate distribution responses to predator type was polymorphic colour vision.

In Chapter 5, a computer vision and machine learning approach was used to infer predator detectability using colour information. We had no intention in recreating the primate vision system in this analysis, but instead only to test whether this information colour emits a detectable visual signal. Conversely, we investigated the colour signals available for predator identification. One recent approach that improves image classification is Convolutional Neural Networks (CNNs) (LeCun, Bengio, & Hinton, 2015). CNNs are much more accurate than the methods employed here; however, it requires a much larger dataset to be built. We found logistical difficulties in creating the training dataset preventing the use of CNN. Although, it is appropriate to the methods employed. Some loss of colour information is expected from using and converting images from RGB to LAB colour space were RAW images would be more suitable. Despite this, the database created from downloaded images allowed the creation of a diverse image dataset with pictures of actual predators, which would be not possible by taking the photographs ourselves.

The limitations of Chapter 6 have been discussed directly in this chapter as it was a proof of concept study.

Despite all the limitations raised here, we believe that the methods and results obtained in this research are valid. The research in this thesis will support future research and makes a significant contribution into the investigation of the importance of polymorphic colour vision in New World primates.

7.1.2 Future research

The research concerning variation in Medium/Long wavelength sensitive opsins would benefit from studies that investigate the whole gene. Methodologies, such as next-generation sequencing, are becoming more accessible (Schuster, 2007). By having longer DNA readings, it is possible to detect the magnitude of nucleotide changes in other sites and allowing opsin sensitivity determination by microspectrophotometry from reconstituted opsins *in vitro* (Hiramatsu et al., 2004; Liebman & Entine, 1964; Yokoyama, 2000). With the full gene sequence and the absorption peaks, it is possible to match variations in the gene and evaluate single nucleotide polymorphisms that affect the photopigment sensitivity in new allelic variations. With a longer gene DNA, it would be possible to proceed with phylogenetic studies to help our understanding of the evolution of colour vision in New World primates. For example, it would be possible to understand if mutated allelic variations are recent or ancient. If opsin variations are newer in the phylogenetic tree, this suggests that environmental pressures are shaping the opsins and the adaptive role of the alleles would be greater. Whereas, if the allelic variations of the Medium/Long wavelength sensitive opsins were ancient the presence or absence in other primate genera would decrease the adaptive value of related phenotypes. Either way, it would be possible to infer the importance of different phenotypes in interacting with the environment.

Studies on the importance of colour vision and its consequences for predator detection are still scarce when compared with studies on food detection (Osorio et al., 2004; Osorio & Vorobyev, 1996; Pessoa et al., 2014). Despite this, the detection of predators is an important aspect in the evolution of colour vision. It is important to proceed with studies that compare the detection abilities of dichromats and trichromats. For instance, we found that trichromats are better at detecting camouflaged predators; however, other studies show that dichromats have advantages in camouflage breaking (Morgan et al., 1992; Saito et al., 2005; Troscianko,

Wilson-Aggarwal, Griffiths, Spottiswoode, & Stevens, 2016). We did not investigate the effects of illumination and future research in this area will help us to understand abilities to detect cryptic targets, such as predators and insect prey, and the cooperation between individuals in a social group in the maintenance of polymorphic colour vision.

Discovering the importance of time of day and light variation, studies on colour constancy with New World primates would help to understand the role of polymorphic colour vision (Maloney & Wandell, 1986). One of the roles of colour constancy is target identification (Hurlbert, 2007). In this sense, it is especially relevant in food and predator identification. Despite the defective colour vision, colour blind humans still display colour constancy to a certain level (Rüttiger, Mayser, Sérey, & Sharpe, 2001). Given the importance of light variation in affecting the behaviour of *Callicebus nigrifrons* (Chapter 2), it would be informative to understand the importance of colour constancy for food and predator identification in this species. To the best of our knowledge, no research has been conducted comparing the colour constancy of trichromatic and dichromatic primates.

Given the potential importance of behavioural cooperation in sharing the advantages of dichromatic and trichromatic colour vision in New World primate groups, the importance of different types of social organisation should be considered. Several New World primate species, such as *Cacajao, Brachyteles,* and callitrichins, present a fission-fusion society, with groups varying in composition (space and time) (Bowler & Bodmer, 2009; Coles, Lee, & Talebi, 2012). This challenges the understanding of complementary advantages of colour vision polymorphism, as groups will often be composed by only dichromatic colour vision individuals. For example, muriquis often travel in male or female only groups (Strier, Mendes, Rímoli, & Rímoli, 1993). Further studies investigating the composition and behaviour of

fission-fusion primate species would be needed to fully understand the cooperation between different phenotypes.

7.2 Conclusion

The high allelic variation for the Medium/Long wavelength sensitive opsin found in this research strongly suggest a trend towards polymorphic colour vision, which results in an increased number of trichromatic females in New World primate groups. By having a group with different phenotypes, niche divergence between male and females is predicted, which was found here in *Callicebus nigriforns*. Light variations in altitude and time of day results in behavioural differences between males and females. Given that females and males differ in the time spent in feeding sites, we conclude foraging advantages exist for trichromatic females. Predators have a significant impact on the geographic richness of primates with a polymorphic colour vision, where a cooperation among individuals with different phenotypes is predicted. For example, trichromatic females spending less time at a feeding site would be able to be more vigilant allowing dichromatic individuals to use the feeding site for longer. The advantage of females in detecting camouflaged predators is also demonstrated here, by analysing the colour patterns in predator through a computer vision algorithm. As variations in light intensities can favour trichromatic individuals, some advantage in dichromatic individuals for target detection is expected. Scotopic vision and ability to break-through camouflage were already reported in the literature. Consequently, this research adds to previous research in establishing proximate cause for polymorphic colour vision in New World primates. We suggest the intricate social behaviour of New World primates as the ultimate causes for polymorphic colour vision.

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Glossary

Alleles – Alternative forms of a gene, in which in this thesis are refers to variants responsible for converting opsins with different peaks of spectral sensitivity.

Catarrhini – Parvorder which includes Old World primates such as orangutans, chimpanzees, macaques, and humans.

Colour vision – Ability to compare different wavelengths of light to generate a colour perception from a source reflecting or emitting light.

Cones – Photoreceptor cell containing opsins responsible for colour vision

Dichromats – Organisms with two classes of photoreceptors

Electroretinogram – Test that measures the electrical activity and responses to light frequencies of cones and rods in the retina

Folivory – Animal with a diet based on leaves

Frugivory – Animal with a diet based on fruits

Hemizygote – Diploid individual carrying only one copy of allele. For instance, one allele of the Medium/Long wavelength opsin gene present in the X sexual chromosome.

Heterozygote – Diploid individual with two different alleles for the same gene. For instance, trichromatic individuals have two different alleles for the Medium/Long wavelength sensitive opsin and one of the short wavelength sensitive opsin.

Homozygote- diploid individual with allele of the same gene. For instance, dichromatic individuals have the same alleles for the Medium/Long wavelength sensitive opsin and one for the short wavelength opsin.

Luminance – Achromatic signal, brightness.

Machine learning – Automatic classification method used for pattern recognition

Monocromats – Individuals with no colour vision who has only one type of photoreceptor.

New World primates – Primates from the parvorder Platyrrhini, present in South and Central America. Includes species such as marmosets, titis, spider monkeys.

Nm - Nanometres

Old World primates – Primates from the parvorder Catarrhine, present in Asia, Middle East, Africa

Opsin – Proteins (Photopigment) that linked to a photosensitive chromophore are responsible for the conversion of light into neurochemical signals to the brain

Photopigment – Protein that changes the conformation in response to light triggering electrochemical signalling to the brain

Photoreceptor – Specialised cells responsible for light detection. Rods and cones.

Platyrrhini – Parvorder of primates from the New World, which includes species such as marmosets, titis and spider monkeys

Polymorphism – Intraspecific variation of a feature. In this thesis refers to the polymorphic colour vision, where the same species can have dichromatic or trichromatic colour vision individuals.

Rods – Photoreceptor cell containing rhodopsins responsible for scotopic vision

Scotopic – Vision in low light levels

Spectrophotometry – Quantitative analysis of the wavelengths reflected or emitted by an object.

Strepsirrhini – Suborder of primate species, which includes lemurs and lorises.

Tetrachromats – Organisms with four classes of photoreceptors

Trichromats – Organisms with three classes of photoreceptors

Appendix I – List of species used for the biogeographic analysis

Bird predators used in this study

Accipiter bicolor (Vieillot, 1817), *Accipiter poliogaster* (Temminck, 1824), *Aquila chrysaetos* (Linnaeus, 1758), *Busarellus nigricollis* (Latham, 1790), *Buteo jamaicensis* (Gmelin, JF, 1788) , *Geranoaetus melanoleucus* (Vieillot, 1819), *Harpia harpyja* (Linnaeus, 1758), *Leptodon cayanensis* (Latham, 1790), *Leucopternis albicollis* (Latham, 1790), *Leucopternis schistaceus* (Sundevall, 1850), *Micrastur semitorquatus* (Vieillot, 1817), *Micrastur ruficollis* (Vieillot, 1817), *Morphnus guianensis* (Daudin, 1800), *Parabuteo unicinctus* (Temminck, 1824), *Spizaetus isidori* (Des Murs, 1845), *Spizaetus melanoleucus* (Vieillot, 1816), *Spizaetus ornatus* (Daudin, 1800), and *Spizaetus tyrannus* (Wied-Neuwied, 1820).

Mammal predators

Panthera onca (Linnaeus, 1758), *Leopardus pardalis* (Linnaeus, 1758), *Leopardus geoffroyi* (d'Orbigny and Gervais, 1844), *Leopardus tigrinus* (Schreber, 1775), *Puma concolor* (Linnaeus, 1771), *Herpailurus yagouaroundi* (É. Geoffroy Saint-Hilaire, 1803), *Leopardus wiedii* (Schinz, 1821), *Leopardus colocolo* (Molina, 1782) and *Eira Barbara* (Linnaeus, 1758)

Appendix II – Colour extraction algorithm

```
function [features, metrics] =colorprocessing(I)

[~,~,P] = size(I);

isColorImage = P == 3;

if isColorImage

    Ilab = rgb2lab(I);

    Ilab = imresize(Ilab, 1/8);
    % Reshape L*a*b* image into "number of features"-by-3 matrix.
    [Mr,Nr,~] = size(Ilab);
    colorFeatures = reshape(Ilab, Mr*Nr, []);

    % L2 normalize color features
    rowNorm = sqrt(sum(colorFeatures.^2,2));
    colorFeatures = bsxfun(@rdivide, colorFeatures, rowNorm + eps);

    % Normalize pixel coordinates to handle different image sizes.
    xnorm = linspace(-0.5, 0.5, Nr);
    ynorm = linspace(-0.5, 0.5, Mr);
    [x, y] = meshgrid(xnorm, ynorm);

    % Concatenate the spatial locations and color features.
    features = [colorFeatures y(:) x(:)];

    % Use color variance as feature metric.
    metrics  = var(colorFeatures(:,1:3),0,2);
else

    % Return empty features for non-color images. These features are
    % ignored by bagOfFeatures.
    features = zeros(0,5);
    metrics  = zeros(0,1);
end
```

Adapted from: https://uk.mathworks.com/help/vision/examples/image-retrieval-using-customized-bag-of-features.html

Appendix III – Code for feature extraction using BagOfFeatures command, Matlab.

```
images= imageSet('Virtual_Primate', 'recursive');

%%
% Create a custom bag of features using the 'CustomExtractor' option
colorBag = bagOfFeatures(images, ...
'CustomExtractor', @colorprocessing, ...
'VocabularySize', 1000);

%%
scenedata = double(encode(colorBag, images));
%%
SceneImageData = array2table(scenedata);
SceneImageData.imageType = categorical(repelem({images.Description}', ...
        [images.Count], 1));

%% Run Classification learner to select the best classifier
```

Appendix IV - Arduino code for wireless activation of the feeder

```
int Motor1 = 2;
int Motor2 = 3;
int state = 0;
int flag=0;

void setup()
{
  // set all the motor control pins to outputs

  pinMode(Motor1, OUTPUT);
  pinMode(Motor2, OUTPUT);

Serial.begin(9600);
}
void loop()
{

    if(Serial.available() > 0){
      state = Serial.read();
      flag=0;
}
if (state == '0') {
        digitalWrite(Motor1, LOW);
        digitalWrite(Motor2, LOW);
        if(flag == 0){
          Serial.println("Motor: off");
          flag=1;
}

}
   else if (state == '1') {
        digitalWrite(Motor1, LOW);
        digitalWrite(Motor2, HIGH);
        delay(500);
        digitalWrite(Motor1,LOW);
        digitalWrite(Motor2,LOW);
        if(flag == 0){
          Serial.println("Motor: ON");
          flag=1;
        }
    }


}
arduino-builder/arduino-builder -compile -core-api-version 10611 -build-
path /tmp/750124652 -hardware arduino-builder/hardware -hardware arduino-
builder/packages/cores -tools arduino-builder/tools -tools arduino-
builder/packages/tools -built-in-libraries arduino-builder/latest -
libraries /tmp/331172097/pinned -libraries /tmp/331172097/custom -fqbn
Intel:arc32:arduino_101 -build-cache /tmp -verbose=false
/tmp/331172097/sketch_may17a

Sketch uses 49156 bytes (31%) of program storage space. Maximum is 155648
bytes.
```

Appendix V – Code for the pseudoisochromatic image

```
e[] circles = new Circle[5000];

String motiv = "blank";

int count = 0;
int maxDiameter = 22;
int minDiameter = 8;
int lastAdded = 0;
int lastAddedTimeout = 100;

PImage motive;

color[] off, on;

void setup() {
  size(1024, 576);
  smooth();
  background(255);
  colorMode(RGB);
  noFill();
  motive = loadImage(motiv+".png");

  color[] _off = {
// style 1
   color(#9CA594), color(#ACB4A5), color(#BBB964), color(#D7DAAA),
color(#E5D57D), color(#D1D6AF)
// style 2
/*     color(#F49427), color(#C9785D), color(#E88C6A), color(#F1B081),
     color(#F49427), color(#C9785D), color(#E88C6A), color(#F1B081),
     color(#F49427), color(#C9785D), color(#E88C6A), color(#F1B081),
color(#FFCE00)*/
  };

  color[] _on = {
    color(#F9BB82), color(#EBA170), color(#FCCD84)
/*    color(#89B270), color(#7AA45E),  color(#B6C674),  color(#7AA45E),
color(#B6C674),
    color(#89B270), color(#7AA45E),  color(#B6C674),  color(#7AA45E),
color(#B6C674),
     color(#89B270), color(#7AA45E),  color(#B6C674),  color(#7AA45E),
color(#B6C674), color(#FECB05)*/
  };

  on = _on;
  off = _off;


}

void draw() {
  if (count < circles.length) {
    circles[count] = new Circle();
    if (circles[count].overlapsAny()) {
      circles[count] = null;
    }

    if (circles[count] != null) {
      circles[count].draw();
```

```
      if (count > 1) {
        float nearest = 100000;
        float current = 0;
        int nearestIndex = -1;
        for (int i=0; i<count; i++) {
          current = dist(circles[i].x, circles[i].y, circles[count].x,
circles[count].y);
          if (current < nearest) {
            nearest = current;
            nearestIndex = i;
          }
        }

      }

      count++;
      lastAdded = 0;
    } else {
      if (lastAdded > lastAddedTimeout && maxDiameter > minDiameter) {
         maxDiameter--;
         // minDiameter--;
         lastAdded = 0;
       }
      lastAdded++;
     }
  }
  lastX = lastY = -1;
}

class Point
{
   float x,y;

   Point(float x, float y) {
     this.x = x;
     this.y = y;
   }
}

class Circle
{
  float x, y, radius;
  int[] xs, ys;
  color bg = color(255,255,255), fg = -1;

  Circle() {
    radius = random(minDiameter, maxDiameter) / 2.0;
    float a = random(PI*2);
    float r = random(0, width*.48-radius);
    x = lastX < 0 ? width*.5+cos(a)*r : lastX;
    y = lastY < 0 ? height*.5+ sin(a)*r : lastY;
    init();
  }

  Circle(int x, int y, float rad) {
    this.radius = rad;
    this.x = x;
    this.y = y;
    init();
  }
```

```
  void init() {
    int x = int(this.x), y = int(this.y), r = int(radius);
    int[] xs = {x,x ,x  ,x-r,x+r,int(x-r*.93),int(x-
r*.93),int(x+r*.93),int(x+r*.93)};
    int[] ys = {y,y-r,y+r,y,y,int(y+r*.93),int(y-r*.93),int(y+r*.93),int(y-
r*.93)};
    this.xs = xs;
```

Adapted from available codes on Github.com